Exhibit 19

(FILE 'HOME' ENTERED AT 12:39:28 ON 29 MAR 2000)

FILE 'MEDLINE, BIOSIS, CAPLUS, EMBASE, SCISEARCH' ENTERED AT 12:39:43 ON 29 MAR 2000

L1	3923051	S	SERUM OR PLASMA
L2	821836	S	PRENATAL OR MATERNAL OR FETAL OR FOETAL
L3	136809	S	L1 AND L2
L4	1820	S	L3 AND (PCR OR NUCLEIC ACID)
L5	11088	S	L3 AND (PCR OR NUCLEIC ACID OR DNA)
L6	4454	S	L5 NOT (CALF OR BOVINE)
L7	1746	S	L6 AND (SERUM/TI OR PLASMA/TI OR PRENATAL/TI OR FETAL/TI OR
L8	749		DUPLICATE REMOVE L7 (997 DUPLICATES REMOVED)
L9	541	S	L8 AND (MATERNAL/TI OR FETAL/TI)

(FILE 'HOME' ENTERED AT 13:49:47 ON 14 FEB 2000)

FILE 'MEDLINE, BIOSIS, CAPLUS, EMBASE, SCISEARCH' ENTERED AT 13:49:56 ON 14 FEB 2000

E LO/AU
E LO D/AU
L1 17 S E24
E LO DENN/AU
E LO YUK/AU
E LO YUK-MI/AU
E WAINSCOAT/AU
L2 727 S E4-E8
L3 744 S L1 OR L2
L4 314 S L3 AND (NUCLEIC ACID OR DNA)
L5 68 S L4 AND (MATERNAL OR FOETAL OR FETAL)
L6 33 DUPLICATE REMOVE L5 (35 DUPLICATES REMOVED)
L7 8 S L6 AND (SERUM OR PLASMA)

L8 727600 S MATERNAL OR FOETAL OR FETAL
L9 3900005 S SERUM OR PLASMA
L10 132294 S L9 AND L8
L11 503524 S PCR OR POLYMERASE CHAIN
L12 2071 S L10 AND L11
L13 515524 S Y OR DYS14 OR SRY OR RHESUS D
L14 50 S L13 AND L12
L15 28 DUPLICATE REMOVE L14 (22 DUPLICATES REMOVED)
L16 251 S L12 AND (SERUM OR PLASMA)/TI
L17 97 DUPLICATE REMOVE L16 (154 DUPLICATES REMOVED)
L18 71 S L17 NOT (CALF OR BOVINE)
L19 28 S L18 AND (DIAGNOS?)

L9 ANSWER 1 OF 541 MEDLINE
ACCESSION NUMBER: 2000125852 MEDLINE
DOCUMENT NUMBER: 20125852
TITLE: ***Prenatal*** diagnosis of myotonic dystrophy using ***fetal*** ***DNA*** obtained from ***maternal*** ***plasma*** .
AUTHOR: Amicucci P; Gennarelli M; Novelli G; Dallapiccola B
CORPORATE SOURCE: Department of Biopathology and Diagnostic Imaging, Tor Vergata University of Rome, Via Di Tor Vergata 135, 00133 Rome, Italy.
SOURCE: CLINICAL CHEMISTRY, (2000 Feb) 46 (2) 301-2.
Journal code: DBZ. ISSN: 0009-9147.
PUB. COUNTRY: United States
(CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 200004
ENTRY WEEK: 20000404

L9 ANSWER 5 OF 541 MEDLINE
ACCESSION NUMBER: 2000054918 MEDLINE
DOCUMENT NUMBER: 20054918
TITLE: Rapid ***prenatal*** diagnosis of aneuploidy by quantitative fluorescent ***PCR*** on ***fetal*** samples from mothers at high risk for chromosome disorders.
AUTHOR: Pertl B; Pieber D; Lercher-Hartlieb A; Orescovic I; Haeusler M; Winter R; Kroisel P; Adinolfi M
CORPORATE SOURCE: Department of Obstetrics and Gynecology, University of Graz, Auenbruggerplatz 14, A-8036 Graz, Austria.
SOURCE: MOLECULAR HUMAN REPRODUCTION, (1999 Dec) 5 (12) 1176-9.
Journal code: CWO. ISSN: 1360-9947.
PUB. COUNTRY: ENGLAND: United Kingdom
(CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200003
ENTRY WEEK: 20000303
AB We report the results of a prospective study using quantitative fluorescent polymerase chain reaction (QF- ***PCR***) and small tandem repeat markers (STR) for the rapid ***prenatal*** detection of aneuploidies in a group of pregnant women at increased risk of having fetuses with numerical chromosome disorders. Amniotic fluid samples (n = 52) were collected from mothers undergoing ***prenatal*** invasive testing for ***fetal*** abnormalities on ultrasonographic examination or abnormal ***maternal*** ***serum*** aneuploidy screening results. All samples were tested by cytogenetic analysis, but rapid diagnoses of aneuploidies were offered and performed using QF- ***PCR*** analysis with several STRs specific for chromosomes 21, 18, 13 and X. All cases with numerical chromosome aberrations involving chromosomes 21, 18 and 13 (n = 8) were correctly diagnosed. Three gonosomal aneuplodies (one 47,XXY and two 45,X) were not detected because they were uninformative for the X markers. Another sample with a deletion (46,XX,7q-), that the

present assay was not designed to detect, was not identified. One sample was heavily contaminated with ***maternal*** blood and the results of the QF- ***PCR*** assays were uninformative. The remaining samples from normal fetuses provided QF- ***PCR*** patterns disomic for chromosomes 21, 18, 13 and X. Our study demonstrates that QF- ***PCR*** is a rapid method for the detection of common numerical chromosome disorders and it may play an important role in ***prenatal*** diagnosis for women at high risk for ***fetal*** aneuploidy.

L9 ANSWER 7 OF 541 MEDLINE
ACCESSION NUMBER: 2000039659 MEDLINE
DOCUMENT NUMBER: 20039659
TITLE: ***Fetal*** RhD genotyping from ***maternal*** ***plasma*** .
AUTHOR: Lo Y M
CORPORATE SOURCE: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Kong Hong Special Administration Region.. loym@cuhk.edu.hk
SOURCE: ANNALS OF MEDICINE, (1999 Oct) 31 (5) 308-12. Ref: 48
Journal code: AMD. ISSN: 0785-3890.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200002
ENTRY WEEK: 20000204
AB The ***prenatal*** diagnosis of ***fetal*** rhesus D (RhD) status is useful for the management of RhD-negative women with partners heterozygous for the RHD gene. Conventional methods for ***prenatal*** ***fetal*** RhD status determination involve invasive procedures such as ***fetal*** blood sampling and amniocentesis. The recent demonstration of the existence of cell-free ***fetal*** ***DNA*** in ***maternal*** ***plasma*** and ***serum*** opens up the possibility of determining ***fetal*** RhD status by analysis of ***maternal*** ***plasma*** or ***serum*** ***DNA*** . This possibility has recently been realized by three independent groups of investigators. This development represents an important step towards the routine application of noninvasive ***fetal*** blood group diagnosis in sensitized pregnancies and may become a model for developing safer noninvasive ***prenatal*** tests for other single-gene disorders.

L9 ANSWER 9 OF 541 MEDLINE
ACCESSION NUMBER: 2000012845 MEDLINE
DOCUMENT NUMBER: 20012845
TITLE: Detection of ***fetal*** -derived paternally inherited X-chromosome polymorphisms in ***maternal*** ***plasma*** .
AUTHOR: Tang N L; Leung T N; Zhang J; Lau T K; Lo Y M
CORPORATE SOURCE: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR.
SOURCE: CLINICAL CHEMISTRY, (1999 Nov) 45 (11) 2033-5.
Journal code: DBZ. ISSN: 0009-9147.

PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 200001
ENTRY WEEK: 20000104

L9 ANSWER 1 OF 541 MEDLINE
ACCESSION NUMBER: 2000125852 MEDLINE
DOCUMENT NUMBER: 20125852
TITLE: ***Prenatal*** diagnosis of myotonic dystrophy using ***fetal*** ***DNA*** obtained from ***maternal*** ***plasma*** .
AUTHOR: Amicucci P; Gennarelli M; Novelli G; Dallapiccola B
CORPORATE SOURCE: Department of Biopathology and Diagnostic Imaging, Tor Vergata University of Rome, Via Di Tor Vergata 135, 00133 Rome, Italy.
SOURCE: CLINICAL CHEMISTRY, (2000 Feb) 46 (2) 301-2.
Journal code: DBZ. ISSN: 0009-9147.
PUB. COUNTRY: United States
(CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 200004
ENTRY WEEK: 20000404

L9 ANSWER 13 OF 541 MEDLINE
ACCESSION NUMBER: 1999422253 MEDLINE
DOCUMENT NUMBER: 99422253
TITLE: ***Foetal*** RhD genotyping using ***DNA*** extracted from ***maternal*** ***plasma*** .
AUTHOR: Mohan A; Seth S
CORPORATE SOURCE: Department of Emergency Medicine, Sir Venkateswara Institute of Medical Science, Tirupati, Andhra Pradesh.
SOURCE: NATIONAL MEDICAL JOURNAL OF INDIA, (1999 May-Jun) 12 (3) 118-9.
Journal code: BNT. ISSN: 0970-258X.
PUB. COUNTRY: India
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
ENTRY MONTH: 199912
ENTRY WEEK: 19991201

L9 ANSWER 14 OF 541 MEDLINE
ACCESSION NUMBER: 1999402887 MEDLINE
DOCUMENT NUMBER: 99402887
TITLE: Evaluation of different approaches for ***fetal*** ***DNA*** analysis from ***maternal*** ***plasma*** and nucleated blood cells.
AUTHOR: Smid M; Lagona F; de Benassuti L; Ferrari A; Ferrari M; Cremonesi L
CORPORATE SOURCE: Istituto di Rivocero e Cura a Carattere Scientifico, Hospital San Raffaele, Department of Obstetrics and Gynecology, Via Olgettina 60, 20132 Milan, Italy.
SOURCE: CLINICAL CHEMISTRY, (1999 Sep) 45 (9) 1570-2.

Journal code: DBZ. ISSN: 0009-9147.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199911
ENTRY WEEK: 19991104

L9 ANSWER 27 OF 541 MEDLINE
ACCESSION NUMBER: 1999222507 MEDLINE
DOCUMENT NUMBER: 99222507
TITLE: Noninvasive determination of ***fetal*** RhD status using ***fetal*** ***DNA*** in ***maternal*** ***serum*** and ***PCR*** .
AUTHOR: Bischoff F Z; Nguyen D D; Marquez-Do D; Moise K J Jr; Simpson J L; Elias S
CORPORATE SOURCE: Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas 77030, USA.. bischoff@bcm.tmc.edu
CONTRACT NUMBER: N01-HD43203 (NICHD)
SOURCE: JOURNAL OF THE SOCIETY FOR GYNECOLOGIC INVESTIGATION, (1999 Mar-Apr) 6 (2) 64-9.
Journal code: CMH. ISSN: 1071-5576.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199908
ENTRY WEEK: 19990802
AB OBJECTIVE: Because ***prenatal*** testing of ***fetal*** RhD status by amniocentesis carries small yet finite risks to the fetus and mother, this study sought to determine whether ***fetal*** ***DNA*** in ***maternal*** ***serum*** could be used to detect ***fetal*** RhD status by polymerase chain reaction (***PCR***). METHODS: A retrospective analysis was made of frozen ***serum*** specimens from 20 sensitized RhD-negative pregnant women (ranging from 15.0 to 36.0 weeks' gestation) who were confirmed by serology at birth to have been carrying RhD-positive fetuses. Eleven ***serum*** specimens from RhD-negative individuals served as controls. ***DNA*** was isolated from ***serum*** and used in two ***PCR*** -based methods to detect a 99 base pair (bp) ***DNA*** fragment specific for the RhD gene and a 113 bp fragment specific for the RhCE gene as control. RESULTS: Overall, in 14 (70%) of 20 RhD-positive fetuses the 99 base pair RhD-specific ***PCR*** product was detected. There was no false positive detection among the 11 control ***serum*** specimens. CONCLUSION: The results illustrate the ability to detect ***fetal*** RhD sequences in ***maternal*** ***serum*** of sensitized women. Moreover, the findings demonstrate that ***fetal*** single-gene disorders can be detected prenatally by using ***DNA*** isolated only from ***maternal*** ***serum*** .

L9 ANSWER 33 OF 541 MEDLINE
ACCESSION NUMBER: 1999132218 MEDLINE
DOCUMENT NUMBER: 99132218
TITLE: Quantitative abnormalities of ***fetal*** ***DNA*** in ***maternal*** ***serum*** in preeclampsia [see comments].

COMMENT: Comment in: Clin Chem 1999 Apr;45(4):451-2
AUTHOR: Lo Y M; Leung T N; Tein M S; Sargent I L; Zhang J; Lau T K; Haines C J; Redman C W
CORPORATE SOURCE: Departments of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR.. loym@cuhk.edu.hk
SOURCE: CLINICAL CHEMISTRY, (1999 Feb) 45 (2) 184-8.
Journal code: DBZ. ISSN: 0009-9147.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Cancer Journals; Priority Journals
ENTRY MONTH: 199904
AB BACKGROUND: There is much recent interest in the biologic and diagnostic implication of cell-free non-host ***DNA*** in the ***plasma*** and ***serum*** of human subjects. To determine if quantitative abnormalities of circulating non-host ***DNA*** may be associated with certain pathologic processes, we used circulating ***fetal*** ***DNA*** in preeclampsia as a model system. METHODS: We studied 20 preeclamptic women and 20 control subjects of comparable gestational age (means, 32 and 33 weeks, respectively). Male ***fetal*** ***DNA*** in ***maternal*** ***serum*** was measured using real-time quantitative ***PCR*** for the SRY gene on the Y chromosome. RESULTS: The imprecision (CV) of the assay was 2.7%. The median circulating ***fetal*** ***DNA*** was increased fivefold in 20 preeclamptic women compared with 20 control pregnant women (381 vs 76 genome-equivalents/mL, P <0.001). CONCLUSIONS: These observations suggest that preeclampsia is associated with disturbances in the liberation and/or clearance mechanisms of circulating ***DNA*** . These results also raise the possibility that measurement of circulating ***DNA*** may prove useful as a marker for the diagnosis and/or monitoring of preeclampsia.

L9 ANSWER 36 OF 541 MEDLINE
ACCESSION NUMBER: 1999115099 MEDLINE
DOCUMENT NUMBER: 99115099
TITLE: Rapid clearance of ***fetal*** ***DNA*** from ***maternal*** ***plasma*** .
AUTHOR: Lo Y M; Zhang J; Leung T N; Lau T K; Chang A M; Hjelm N M
CORPORATE SOURCE: Department of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong.
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1999 Jan) 64 (1) 218-24.
Journal code: 3IM. ISSN: 0002-9297.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199905
ENTRY WEEK: 19990502
AB ***Fetal*** ***DNA*** has been detected in ***maternal*** ***plasma*** during pregnancy. We investigated the clearance of circulating ***fetal*** ***DNA*** after delivery, using quantitative ***PCR*** analysis of the sex-determining region Y gene as a marker for male fetuses. We analyzed ***plasma*** samples from 12

women 1-42 d after delivery of male babies and found that circulating ***fetal*** ***DNA*** was undetectable by day 1 after delivery. To obtain a higher time-resolution picture of ***fetal*** ***DNA*** clearance, we performed serial sampling of eight women, which indicated that most women (seven) had undetectable levels of circulating ***fetal*** ***DNA*** by 2 h postpartum. The mean half-life for circulating ***fetal*** ***DNA*** was 16.3 min (range 4-30 min). ***Plasma*** nucleases were found to account for only part of the clearance of ***plasma*** ***fetal*** ***DNA*** . The rapid turnover of circulating ***DNA*** suggests that ***plasma*** ***DNA*** analysis may be less susceptible to false-positive results, which result from carryover from previous pregnancies, than is the detection of ***fetal*** cells in ***maternal*** blood; also, rapid turnover may be useful for the monitoring of feto- ***maternal*** events with rapid dynamics. These results also may have implications for the study of other types of nonhost ***DNA*** in ***plasma*** , such as circulating tumor-derived and graft-derived ***DNA*** in oncology and transplant patients, respectively.

L9 ANSWER 41 OF 541 MEDLINE
ACCESSION NUMBER: 1999049885 MEDLINE
DOCUMENT NUMBER: 99049885
TITLE: ***Prenatal*** diagnosis of ***fetal*** RhD status by molecular analysis of ***maternal*** ***plasma*** [see comments].
COMMENT: Comment in: N Engl J Med 1998 Dec 10;339(24):1775-7
AUTHOR: Lo Y M; Hjelm N M; Fidler C; Sargent I L; Murphy M F; Chamberlain P F; Poon P M; Redman C W; Wainscoat J S
CORPORATE SOURCE: Department of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital.
SOURCE: NEW ENGLAND JOURNAL OF MEDICINE, (1998 Dec 10) 339 (24) 1734-8.
Journal code: NOW. ISSN: 0028-4793.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199902
ENTRY WEEK: 19990204
AB BACKGROUND: The ability to determine ***fetal*** RhD Status noninvasively is useful in the treatment of RhD-sensitized pregnant women whose partners are heterozygous for the RhD gene. The recent demonstration of ***fetal*** ***DNA*** in ***maternal*** ***plasma*** raises the possibility that ***fetal*** RhD genotyping may be possible with the use of ***maternal*** ***plasma*** . METHODS: We studied 57 RhD-negative pregnant women and their singleton fetuses. ***DNA*** extracted from ***maternal*** ***plasma*** was analyzed for the RhD gene with a fluorescence-based polymerase-chain-reaction (***PCR***) test sensitive enough to detect the RhD gene in a single cell. ***Fetal*** RhD status was determined directly by serologic analysis of cord blood or ***PCR*** analysis of amniotic fluid. RESULTS: Among the 57 RhD-negative women, 12 were in their first trimester of pregnancy, 30 were in their second trimester, and 15 were in their third trimester. Thirty-nine fetuses were RhD-positive, and 18 were RhD-negative. In the samples obtained from women in their second or third trimester of

pregnancy, the results of RhD ***PCR*** analysis of ***maternal*** ***plasma*** ***DNA*** were completely concordant with the results of serologic analysis. Among the ***maternal*** ***plasma*** samples collected in the first trimester, 2 contained no RhD ***DNA*** , but the fetuses were RhD-positive; the results in the other 10 samples were concordant (7 were RhD-positive, and 3 RhD-negative). CONCLUSIONS: Noninvasive ***fetal*** RhD genotyping can be performed rapidly and reliably with the use of ***maternal*** ***plasma*** beginning in the second trimester of pregnancy.

L9 ANSWER 44 OF 541 MEDLINE
ACCESSION NUMBER: 1998449274 MEDLINE
DOCUMENT NUMBER: 98449274
TITLE: Detection of ***fetal*** RHD-specific sequences in ***maternal*** ***plasma*** [letter].
AUTHOR: Faas B H; Beuling E A; Christiaens G C; von dem Borne A E; van der Schoot C E
SOURCE: LANCET, (1998 Oct 10) 352 (9135) 1196.
Journal code: L0S. ISSN: 0140-6736.
PUB. COUNTRY: ENGLAND: United Kingdom
Letter
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199901
ENTRY WEEK: 19990104

L9 ANSWER 56 OF 541 MEDLINE
ACCESSION NUMBER: 1998198334 MEDLINE
DOCUMENT NUMBER: 98198334
TITLE: Quantitative analysis of ***fetal*** ***DNA*** in ***maternal*** ***plasma*** and ***serum*** : implications for noninvasive ***prenatal*** diagnosis.
AUTHOR: Lo Y M; Tein M S; Lau T K; Haines C J; Leung T N; Poon P M; Wainscoat J S; Johnson P J; Chang A M; Hjelm N M
CORPORATE SOURCE: Department of Chemical Pathology, The University of Hong Kong, Prince Wales Hospital, Shatin, New Territories, Hong Kong.. loym@cuhk.edu.hk
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1998 Apr) 62 (4) 768-75.
Journal code: 3IM. ISSN: 0002-9297.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199808
ENTRY WEEK: 19980802
AB We have developed a real-time quantitative ***PCR*** assay to measure the concentration of ***fetal*** ***DNA*** in ***maternal*** ***plasma*** and ***serum*** . Our results show that ***fetal*** ***DNA*** is present in high concentrations in ***maternal*** ***plasma*** , reaching a mean of 25.4 genome equivalents/ml (range 3.3-69. 4) in early pregnancy and 292.2 genome equivalents/ml (range 76. 9-769) in late pregnancy. These concentrations correspond to 3.4% (range 0.39%-11.9%) and 6.2% (range 2.33%-11.4%) of the total ***plasma*** ***DNA*** in early and late pregnancy, respectively. Sequential

follow-up study of women who conceived by in vitro fertilization shows that ***fetal*** ***DNA*** can be detected in ***maternal*** ***serum*** as early as the 7th wk of gestation and that it then increases in concentration as pregnancy progresses. These data suggest that ***fetal*** ***DNA*** can be readily detected in ***maternal*** ***plasma*** and ***serum*** and may be a valuable source of material for noninvasive ***prenatal*** diagnosis.

L9 ANSWER 57 OF 541 MEDLINE
ACCESSION NUMBER: 1998198332 MEDLINE
DOCUMENT NUMBER: 98198332
TITLE: ***Fetal*** ***DNA*** in ***maternal*** ***plasma*** : the plot thickens and the placental barrier thins [editorial].
AUTHOR: Bianchi D W
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1998 Apr) 62 (4) 763-4. Ref: 13
Journal code: 3IM. ISSN: 0002-9297.
PUB. COUNTRY: United States
Editorial
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199808
ENTRY WEEK: 19980802

L9 ANSWER 74 OF 541 MEDLINE
ACCESSION NUMBER: 97420079 MEDLINE
DOCUMENT NUMBER: 97420079
TITLE: Presence of ***fetal*** ***DNA*** in ***maternal*** ***plasma*** and ***serum*** .
AUTHOR: Lo Y M; Corbetta N; Chamberlain P F; Rai V; Sargent I L; Redman C W; Wainscoat J S
CORPORATE SOURCE: Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, University of Oxford, UK.
SOURCE: LANCET, (1997 Aug 16) 350 (9076) 485-7.
Journal code: L0S. ISSN: 0140-6736.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals; Cancer Journals
ENTRY MONTH: 199711
ENTRY WEEK: 19971103
AB BACKGROUND: The potential use of ***plasma*** and ***serum*** for molecular diagnosis has generated interest. Tumour ***DNA*** has been found in 'the ***plasma*** and ***serum*** of cancer patients, and molecular analysis has been done on this material. We investigated the equivalent condition in pregnancy-that is, whether ***fetal*** ***DNA*** is present in ***maternal*** ***plasma*** and ***serum*** . METHODS: We used a rapid-boiling method to extract ***DNA*** from ***plasma*** and ***serum*** . ***DNA*** from ***plasma*** , ***serum*** , and nucleated blood cells from 43 pregnant women underwent a sensitive Y- ***PCR*** assay to detect circulating male ***fetal*** ***DNA*** from women bearing male

fetuses. FINDINGS: Fetus-derived Y sequences were detected in 24 (80%) of the 30 ***maternal*** ***plasma*** samples, and in 21 (70%) of the 30 ***maternal*** ***serum*** samples, from women bearing male fetuses. These results were obtained with only 10 microL of the samples. When ***DNA*** from nucleated blood cells extracted from a similar volume of blood was used, only five (17%) of the 30 samples gave a positive Y signal. None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had positive results for ***plasma*** , ***serum*** or nucleated blood cells. INTERPRETATION: Our finding of circulating ***fetal*** ***DNA*** in ***maternal*** ***plasma*** may have implications for non-invasive ***prenatal*** diagnosis, and for improving our understanding of the fetomaternal relationship.

PATENT APPLICATION FEE DETERMINATION RECORD

Effective November 10, 1998

Application or Docket Number: 09/380696

CLAIMS AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA
BASIC FEE		
TOTAL CLAIMS	26 minus 20=	* 6
INDEPENDENT CLAIMS	3 minus 3 =	*
MULTIPLE DEPENDENT CLAIM PRESENT		

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐ RATE	FEE	OR	OTHER THAN SMALL ENTITY RATE	FEE
420	~~380.00~~	OR		840
X$ 9=	54	OR	X$18=	108
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL	474	OR	TOTAL	948

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
13				
Total	* 27	Minus	** 26	= 1
Independent	* 3	Minus	*** 3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	18
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	paid

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

(FOR USE WITH FORM PTO-875)

SERIAL NO.

FILING DATE

APPLICANT(S) 09/380696

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT			*		*		*	
	IND.	DEP.	IND.	DEP.	IND.	DEP.		IND.	DEP.	IND.	DEP.	IND.	DEP.
1	1						51						
2		1					52						
3		1					53						
4		01					54						
5		01					55						
6		01					56						
7		01					57						
8		01					58						
9		01					59						
10		01					60						
11		01					61						
12		01					62						
13		01					63						
14		01					64						
15		01					65						
16		01					66						
17		01					67						
18		01					68						
19		01					69						
20		01					70						
21		01					71						
22		01					72						
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excretion and that loss of glomerular anionic content may be associated with increased urinary GAG excretion.

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Prenatal Diagnosis of Myotonic Dystrophy Using Fetal DNA Obtained from Maternal Plasma, *Paola Amicucci,[1,2] Massimo Gennarelli,[3] Giuseppe Novelli,[1,2]* and Bruno Dallapiccola[1,2]* ([1] Department of Biopathology and Diagnostic Imaging, Tor Vergata University of Rome, Via Di Tor Vergata 135, 00133 Rome, Italy; [2] CSS-Mendel, Piazza Galeno 3, 00161 Rome, Italy; [3] Istituto di Ricovero e Cura a Carattere Scientifico, Fatebenefratelli, Via Pilastroni 4, 25125 Brescia, Italy; * author for correspondence: fax 39-06-20427313, e-mail novelli@med.uniroma2.it)

Myotonic dystrophy (DM; MIM 160900) is an autosomal dominant disorder associated with expansion of an unstable CTG trinucleotide repeat in the 3′ untranslated region of the DM kinase gene (*DMPK*) on chromosome 19q13 *(1)*. Patients are heterozygous for expanded alleles in the range of 50–4000 repeats *(1)*. The molecular diagnosis of DM routinely is performed by analyzing the CTG number on genomic DNA extracted from various biological sources, including trophoblast cells sampled at 10–11 weeks of amenorrhea during the first trimester of pregnancy *(2, 3)*. We evaluated the possibility of using maternal plasma for prenatal diagnosis of DM, by monitoring the pregnancy of an unaffected woman whose husband was affected by DM (70 CTG repeats).

All participants gave oral and written informed consent.

A blood sample (~10 mL) was collected at 10 weeks of gestation before chorionic villus sampling (CVS) and was centrifuged at 3000*g* for 10 min. Plasma was carefully removed from EDTA-containing tube and centrifuged again at 3000*g* for 10 min. DNA was then extracted from 2 mL of the centrifuged plasma with a QIAamp Blood Kit (Qiagen). The elution volume of the final step was 300 μL. Genomic DNA was also extracted from chorionic villi and peripheral blood lymphocytes of both parents.

To check for the presence of fetal DNA in maternal plasma, we performed microsatellite DNA analysis (CSF1PO) and Y-specific PCR (amelogenin) amplification after having ascertained that the fetus was a male (Fig. 1, A and B). *DMPK* CTG repeat amplification was carried out as reported previously *(2)* with a slight modification. A first round of PCR consisting of 15 cycles (30 s at 94 °C, 1 min at 62 °C, 5 min at 68 °C, and a final elongation of 5 min at 68 °C), was performed in 30 μL of reaction mixture, using 25 pmol each of forward and reverse primers DMK9003 (5′-CACAGGCTGAAGTGGCAGTTCCA-3′) and DMK11111 (5′-TGTCGGGGTCTCAGTGCATCCA-3′) *(2)*, and 5–10 μL of the extracted DNA. We reamplified 1 μL of this first-round reaction, using 25 pmol each of forward and reverse primers MDY-1D (5′-GCTCGAAGGGTCCTTGTAGCCG-3′) and MDY-Z2A (5′-TTCCCGAGTAAGCAGGCAGA-3′) *(3)* for 40 additional cycles, using the same cycling and reaction conditions. Amplicons were separated by 1% agarose gel electrophoresis and blotted onto a nylon membrane. Filters were hybridized with $(CTG)_5$ ^{32}P-labeled oligonucleotide as described *(3)*. The same protocol was used for genomic

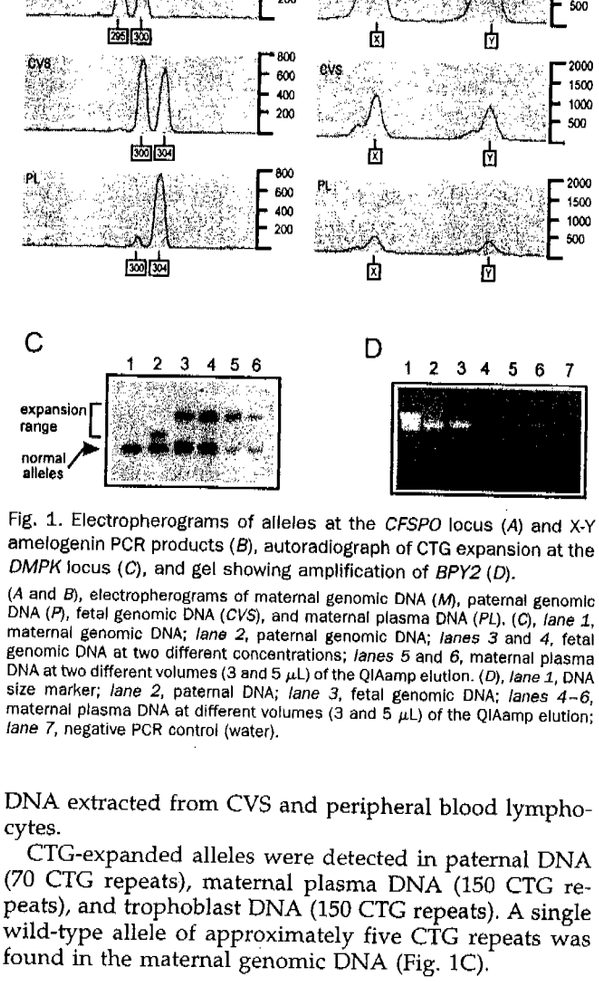

Fig. 1. Electropherograms of alleles at the *CFSPO* locus (*A*) and X-Y amelogenin PCR products (*B*), autoradiograph of CTG expansion at the *DMPK* locus (*C*), and gel showing amplification of *BPY2* (*D*).

(*A* and *B*), electropherograms of maternal genomic DNA (*M*), paternal genomic DNA (*P*), fetal genomic DNA (*CVS*), and maternal plasma DNA (*PL*). (*C*), *lane 1*, maternal genomic DNA; *lane 2*, paternal genomic DNA; *lanes 3* and *4*, fetal genomic DNA at two different concentrations; *lanes 5* and *6*, maternal plasma DNA at two different volumes (3 and 5 μL) of the QIAamp elution. (*D*), *lane 1*, DNA size marker; *lane 2*, paternal DNA; *lane 3*, fetal genomic DNA; *lanes 4–6*, maternal plasma DNA at different volumes (3 and 5 μL) of the QIAamp elution; *lane 7*, negative PCR control (water).

DNA extracted from CVS and peripheral blood lymphocytes.

CTG-expanded alleles were detected in paternal DNA (70 CTG repeats), maternal plasma DNA (150 CTG repeats), and trophoblast DNA (150 CTG repeats). A single wild-type allele of approximately five CTG repeats was found in the maternal genomic DNA (Fig. 1C).

To demonstrate that large, CTG-expanded *DMPK* alleles (up to 2000 CTGs) can be detected in maternal plasma, we performed a long-PCR to amplify an 8-kilobase DNA fragment of the basic protein Y2 (*BPY2*) gene mapping to the Y-chromosome (Fig. 1D). PCR consisting of 35 cycles (2 min at 94 °C, 30 s at 65 °C, 6 min at 68 °C, and a final elongation of 5 min at 68 °C) was performed in 30 μL of reaction mixture using 25 pmol each of forward and reverse primers 7R (5′-GGTATCTGAAGCTGGGTATATGAC-3′) and 7F (5′-AGATAACATCCATCGTGGCTCTG-3′; A. Pizzuti, unpublished data), and 5–10 μL of plasma extracted DNA.

These results support the possibility of performing prenatal diagnosis of DM with maternal plasma. At present, this test seems appropriate only for monitoring paternally inherited expanded alleles. Noninvasive DM prenatal diagnosis was reported previously by our group on trophoblast cells retrieved from the lower part of the uterine cavity (*4*). However, the amount of fetal DNA recovered with that procedure is low compared with the amount of fetal DNA recovered from maternal plasma (*4–6*). We conclude that this noninvasive method, which allows first-trimester DM prenatal diagnosis using maternal plasma, has the potential to become an alternative procedure in selected cases.

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#2

Noninvasive Determination of Fetal RhD Status Using Fetal DNA in Maternal Serum and PCR

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OBJECTIVE: *Because prenatal testing of fetal RhD status by amniocentesis carries small yet finite risks to the fetus and mother, this study sought to determine whether fetal DNA in maternal serum could be used to detect fetal RhD status by polymerase chain reaction (PCR).*

METHODS: *A retrospective analysis was made of frozen serum specimens from 20 sensitized RhD-negative pregnant women (ranging from 15.0 to 36.0 weeks' gestation) who were confirmed by serology at birth to have been carrying RhD-positive fetuses. Eleven serum specimens from RhD-negative individuals served as controls. DNA was isolated from serum and used in two PCR-based methods to detect a 99 base pair (bp) DNA fragment specific for the RhD gene and a 113 bp fragment specific for the RhCE gene as control.*

RESULTS: *Overall, in 14 (70%) of 20 RhD-positive fetuses the 99 base pair RhD-specific PCR product was detected. There was no false positive detection among the 11 control serum specimens.*

CONCLUSION: *The results illustrate the ability to detect fetal RhD sequences in maternal serum of sensitized women. Moreover, the findings demonstrate that fetal single-gene disorders can be detected prenatally by using DNA isolated only from maternal serum. (J Soc Gynecol Invest 1999;6:64–9)*

KEY WORDS: Fetal RhD, noninvasive prenatal diagnosis, maternal serum DNA, polymerase chain reaction.

The RhD antigen is highly immunogenic and responsible for the majority of cases of Rh-alloimmunization.[1] Approximately 55% of the RhD-positive white population is heterozygous for the presence of the RhD gene.[2] The fetus of an RhD-negative woman with an RhD-positive heterozygous partner has a 50% chance of being RhD-negative and a 50% chance of being heterozygous RhD-positive. In the latter situation, if the mother has been sensitized from a previous RhD-positive pregnancy, the fetus may be affected with severe hemolytic disease, which can lead to fetal hydrops and intrauterine fetal death. Therefore, prenatal determination of fetal RhD status early in pregnancy is important for the management of pregnancies involving sensitized RhD-negative women.

The Rh blood group has been mapped to chromosome 1 (1p34-36) and contains two homologous genes.[3,4] One gene encodes for the D antigen, which is present only on the erythrocyte membrane of Rh-positive individuals.[5] The other gene encodes for the C/c and E/e polypeptides present on red blood cells of all individuals.[5] Antisera to five Rh antigens (D, C, c, E, and e) are available and are used to type individuals serologically. In general, the RhD gene is deleted, and the D polypeptide is absent in persons serologically typed as RhD-negative. For prenatal determination of fetal RhD status, methods based on the polymerase chain reaction (PCR) have been developed to test for the presence or absence of RhD gene-specific sequences.[6–12] Currently, prenatal testing of fetal RhD status is achieved through amniocentesis, an invasive procedure that usually is performed late in pregnancy and carries small yet finite risks (0.5%) to the fetus and mother.[13] Clearly, a noninvasive prenatal test to determine fetal RhD status early in pregnancy is desirable.

Studies have now shown that fetal DNA exists in maternal serum and plasma.[14,15] In a study of 30 pregnant women with male fetuses, Lo et al[14] reported detection of fetal Y-chromosomal sequences in maternal serum (70%) and plasma (80%) using PCR. More recently, the concentration of fetal DNA in maternal serum has been quantitated and found to comprise 0.13% and 1.0% of the total maternal serum DNA during early and late pregnancy, respectively.[15] The observation of fetal DNA in maternal serum provides evidence for the feasibility of noninvasive prenatal genetic diagnosis of single-gene disorders

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Table 1. Detection of RhD and RhCE Gene Sequences in Maternal Serum DNA by PCR

Case	Gestational Age (wk)	Maternal Anti-D Titer	RhD/RhCE Gene Detection*		Overall Detection§
			F-PCR†	C-PCR‡	
1	20.5	1:256	+/+	−/+	+
2	23.6	1:64	−/+	−/+	−
3	22.2	1:2048	−/+	−/+	−
4	20.9	1:256	+/+	+/+	+
5	21.1	1:512	ins	+/+	+
6	18.2	1:512	+/+	+/+	+
7	22.4	1:32	+/+	−/+	+
8	21.8	1:2048	+/+	+/+	+
9	21.1	1:2048	+/+	−/+	+
10	19.7	1:128	+/+	−/+	+
11	23.0	1:128	ins	+/+	+
12	22.0	1:2048	−/+	−/+	−
13	33.4	1:512	+/+	+/+	+
14	32.3	1:2048	ins	+/+	+
15	36.0	1:512	++	++	+
16	28.9	1:256	−/+	−/+	−
17	33.4	1:1024	−/+	−/+	−
18	26.5	1:256	−/+	−/+	−
19	28.0	1:512	+/+	+/+	+
20	15.0	1:16	ins	+/+	+
Total			10/16 (62%)	10/20 (50%)	14/20 (70%)

PCR = polymerase chain reaction; ins = cases in which fluorescent PCR was not performed owing to insufficient amount of serum; + = positive detection of the gene-specific PCR product; − = no detection of the gene-specific PCR product.
* Simultaneous PCR amplification of the RhD (99 bp) and RhCE (113 bp) genes.
† Amplification by fluorescent PCR method
‡ Amplification by conventional PCR method.
§ Overall detection of RhD gene by fluorescent and/or conventional PCR methods.

in which the inherited mutation or polymorphism is paternally transmitted. In the current study, we focus on the detection of the paternal RhD gene in pregnancies at risk for Rh hemolytic disease.

To test the hypothesis that prenatal genetic diagnosis could be performed by using maternal serum DNA, we initiated a retrospective study to investigate whether fetal RhD status can be determined in sensitized RhD-negative pregnant women with RhD heterozygous partners. We evaluated efficiency in detecting fetal RhD status using two PCR-based methods to amplify simultaneously the RhD and RhCE genes. Our results demonstrate the ability to perform noninvasive genetic detection of fetal RhD status. Moreover, we demonstrate that prenatal genetic diagnosis of certain single-gene disorders can be performed by using maternal serum DNA.

MATERIALS AND METHODS

Serum Specimens

Retrospectively, frozen serum specimens from 20 sensitized RhD-negative pregnant women who were confirmed by serology at birth to have had RhD-positive fetuses were analyzed. Mean gestational age at the time of peripheral blood sampling was 24.5 weeks, ranging from 15.0 to 36.0 weeks' gestation (cases 1–20, Table 1). Maternal anti-D titer level for each case is shown in Table 1. As a positive control, fresh serum from a confirmed RhD-positive nonpregnant woman was collected. In addition, serum was processed from 11 serologically confirmed RhD-negative individuals, including a sensitized RhD-negative woman pregnant with an RhD-negative fetus (1:128 maternal anti-D titer), 4 RhD-negative nonpregnant women, and 6 RhD-negative men. The study was approved by the Baylor College of Medicine Institutional Review Board, and all patients gave written consent.

Sample Preparation

Samples of 8–10 mL of peripheral blood were drawn into vacutainers containing no anticoagulant and allowed to sit at room temperature for 2–3 hours. Clotted blood samples were centrifuged at 2500 rpm; serum was removed and transferred to a 10-mL polypropylene tube. Frozen serum samples were thawed at room temperature and then stored on ice. Serum DNA was isolated as previously described.[14] Briefly, for the frozen and fresh serum samples, 0.4 mL was transferred to a sterile 0.5-mL microcentrifuge tube, immediately heated at 98C for 10 minutes on a heating block, and then centrifuged at 12,000 rpm for 20 minutes. The clear supernatant containing DNA ("boiled serum") was removed and transferred to a new 0.5-mL microcentrifuge tube and stored at 4C.

Polymerase Chain Reaction

Two PCR-based methods were employed. In the first, conventional PCR was followed by ethidium bromide-stained agarose gel electrophoresis. A nested PCR amplification to detect RhD and RhCE genes was performed as previously described by Van den Veyver et al.[11] The outer reaction amplified exon 7 of both genes using one primer pair, RhEX7F and RhEX7R. The inner reaction involved a duplex

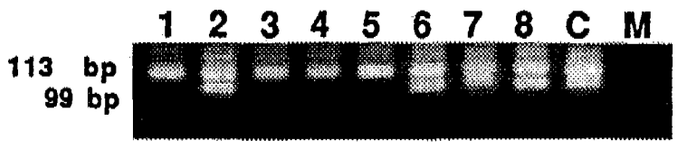

Figure 1. Conventional PCR amplification of RhD and RhCE sequences from maternal serum DNA of RhD-negative women confirmed to have an RhD-positive fetus. RhCE-specific 113-bp fragment is detected in eight representative maternal cases *(lanes 1–8)* and in the RhD-positive serum DNA control (C). RhD-specific 99-bp fragment is detected in four of eight cases shown *(lanes 2, 6–8)* and the control. C = Control serum DNA from a serologically confirmed RhD-positive nonpregnant woman (case 21); M = marker, 100-bp molecular size marker.

amplification using two different primer sets (D969F, D1048R and CE964F, CE1057R) to simultaneously amplify 99-base pair (bp) RhD-specific and 113-bp RhCE-specific fragments. The 113-bp RhCE fragment served as an internal control. Reaction mixtures were prepared exactly as described using 10 μL of serum DNA. The PCR amplification conditions were modified by increasing the number of the inner and outer cycles to 30 and 40, respectively; PCR products were separated on a 3% ethidium bromide-stained agarose gel and visualized under ultraviolet light (Figure 1). To control for reagent contamination and PCR carryover, PCR blanks (reactions containing all the reagents with the exception of DNA) were processed in every experiment.

During this study, a second PCR method, which involved the use of fluorescently labeled primers, became available. Analysis of fluorescent PCR products was accomplished with the ABI PRISM 310 Genetic Analyzer and GeneScan Analysis Software (Perkin-Elmer, Foster City, CA).[16] This is a fully automated genetic analysis system used for sizing PCR or other DNA fragments; it enables multicolor fluorescence technology through the use of an internal size standard. Because of the 1000-fold increased sensitivity of this approach as compared with conventional PCR,[17] only the inner duplex amplification was performed. The PCR conditions were the same as described for the conventional PCR, with the exception that the forward primer of each of the two primer pairs was labeled with a different fluorescent dye. For identification of the RhD-specific PCR product, the D969F primer was labeled with NED (fluoroscein benzoxanthene) and appeared black on the ABI PRISM 310 Genetic Analyzer (Figure 2). Similarly, the CE964F primer was labeled with 6-FAM (6-carboxyfluorescein) and appeared blue (shown as gray) on the ABI PRISM 310 Genetic Analyzer, indicating amplification of the RhCE gene (Figure 2). Because the quantity of frozen serum available for DNA extraction was limited, 1-, 3-, or 10-μL serum DNA aliquots were used for PCR, with the volume of the reaction mixtures adjusted accordingly. Following 60 cycles of amplification, 1 μL of each PCR product was combined with 11.5 μL of deionized formamide and 0.5 μL of GENESCAN-500 size standard labeled with ROX (6-carboxy-X-rhodamine; Perkin-Elmer), which appears red. To control for possible contamination, PCR blanks were also processed in every experiment. The sample mixtures were heated at 94C for 2 minutes, cooled on ice, and then placed on the ABI PRISM 310 Genetic Analyzer sample tray for computer-automated analysis.

RESULTS

PCR Analysis of Maternal Serum DNA

Overall, in 14 (70%) of 20 RhD-positive fetal cases, the 99-bp RhD-specific PCR product was detected (Table 1). In the remaining 6 cases, there was no detection of the RhD-specific sequences by either the conventional or the fluorescent PCR method. The internal control RhCE PCR fragment was detected in all cases by both methods. With conventional PCR, fetal RhD was detected in 10 (50%) of 20 cases (Table 1, Figure 1). In the samples analyzed by fluorescent PCR, serum DNA was available only in 16 of the 20 cases; among these 16 cases, the RhD gene was detected in 10 (62%) (Table 1, Figure 2A). In 4 cases (cases 1, 7, 9, 10) positive detection of the RhD gene was achieved with only the fluorescent-PCR method; conventional PCR failed to detect the RhD gene in these cases.

PCR Analysis of Control Serum DNA

No false positives were detected among the 11 RhD-negative control cases. In the one case of a sensitized RhD-negative woman pregnant with an RhD-negative fetus, both PCR methods were used and detected only the RhCE PCR fragment. Similarly, only the RhCE gene was detected in the 10 remaining negative control cases.

Sensitivity of PCR-Based Methods

To address the sensitivity of the PCR methods, spiked serum samples were prepared by mixing 50, 25, 10, 1, 0.5, and 0.05% (vol/vol) RhD-negative serum into RhD-positive serum. The RhD- and RhCE-specific fragments were detected in all the spiked samples by both PCR methods. Figure 2B illustrates fluorescent PCR detection of the RhD-specific product (black peak) in the 10, 1, 0.5, and 0.05% spiked serum samples.

DISCUSSION

We have illustrated that fetal RhD sequences can be detected by PCR when using maternal serum DNA from sensitized women. Our overall observed 70% (Table 1) detection rate is equivalent to the previously reported 70% detection of fetal chromosomal Y-specific sequences in serum from women confirmed to be carrying a male fetus.[14] In the remaining 30%

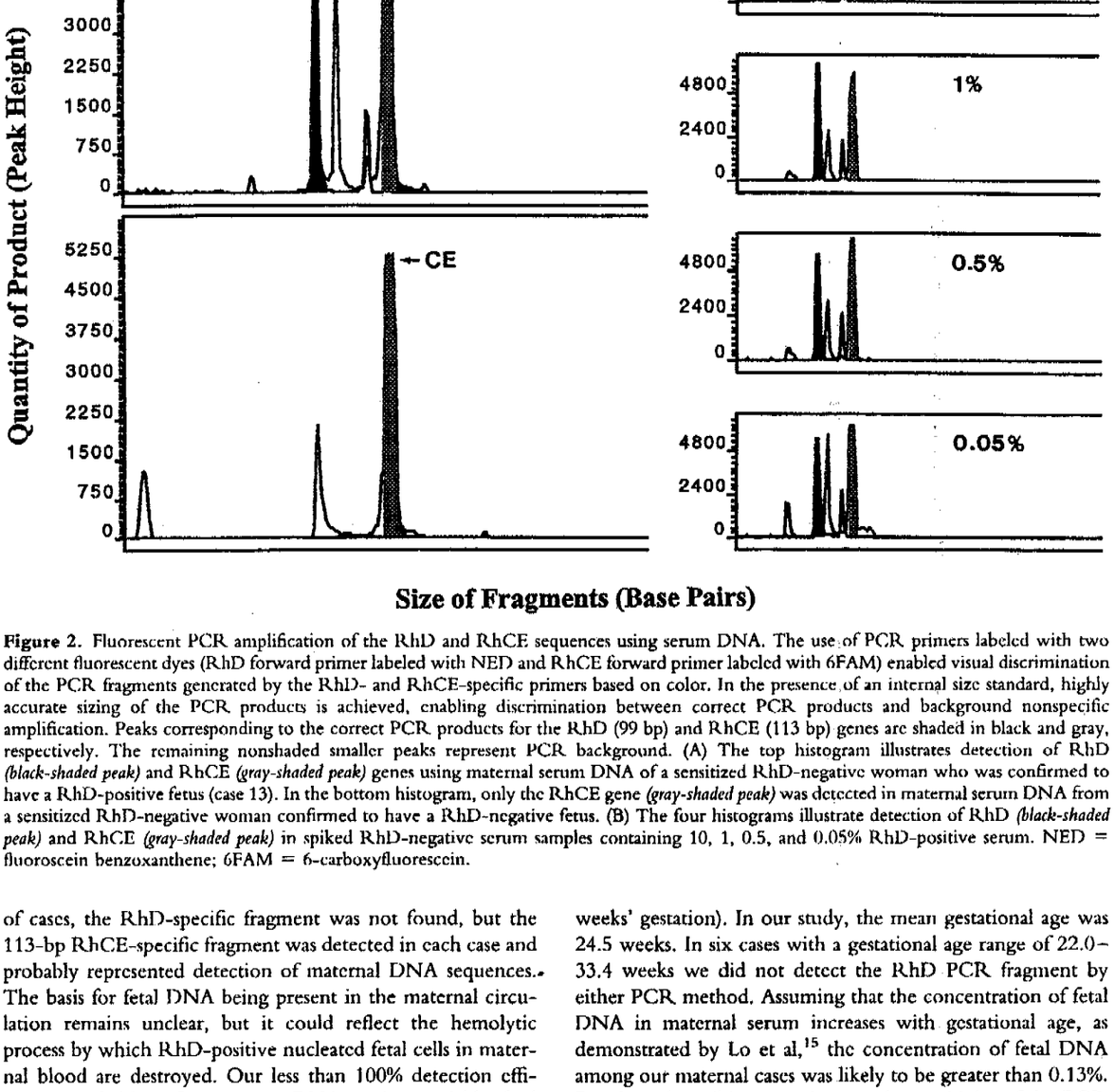

Figure 2. Fluorescent PCR amplification of the RhD and RhCE sequences using serum DNA. The use of PCR primers labeled with two different fluorescent dyes (RhD forward primer labeled with NED and RhCE forward primer labeled with 6FAM) enabled visual discrimination of the PCR fragments generated by the RhD- and RhCE-specific primers based on color. In the presence of an internal size standard, highly accurate sizing of the PCR products is achieved, enabling discrimination between correct PCR products and background nonspecific amplification. Peaks corresponding to the correct PCR products for the RhD (99 bp) and RhCE (113 bp) genes are shaded in black and gray, respectively. The remaining nonshaded smaller peaks represent PCR background. (A) The top histogram illustrates detection of RhD *(black-shaded peak)* and RhCE *(gray-shaded peak)* genes using maternal serum DNA of a sensitized RhD-negative woman who was confirmed to have a RhD-positive fetus (case 13). In the bottom histogram, only the RhCE gene *(gray-shaded peak)* was detected in maternal serum DNA from a sensitized RhD-negative woman confirmed to have a RhD-negative fetus. (B) The four histograms illustrate detection of RhD *(black-shaded peak)* and RhCE *(gray-shaded peak)* in spiked RhD-negative serum samples containing 10, 1, 0.5, and 0.05% RhD-positive serum. NED = fluoroscein benzoxanthene; 6FAM = 6-carboxyfluorescein.

of cases, the RhD-specific fragment was not found, but the 113-bp RhCE-specific fragment was detected in each case and probably represented detection of maternal DNA sequences. The basis for fetal DNA being present in the maternal circulation remains unclear, but it could reflect the hemolytic process by which RhD-positive nucleated fetal cells in maternal blood are destroyed. Our less than 100% detection efficiency probably reflects serum DNA purity, variable fetal DNA concentration in maternal serum, DNA degradation caused by freezing and thawing of the serum samples, or some combination of the three. In a recent study of 27 women pregnant with male fetuses, Lo et al[15] reported fetal DNA concentration in maternal serum to range from 0.014 to 0.54% (mean, 0.13%) in early pregnancy (11–17 weeks' gestation) and from 0.032 to 3.9% (mean, 1.0%) in late pregnancy (37–43 weeks' gestation). In our study, the mean gestational age was 24.5 weeks. In six cases with a gestational age range of 22.0–33.4 weeks we did not detect the RhD PCR fragment by either PCR method. Assuming that the concentration of fetal DNA in maternal serum increases with gestational age, as demonstrated by Lo et al,[15] the concentration of fetal DNA among our maternal cases was likely to be greater than 0.13%. Based on the spiking experiments, the sensitivity of the PCR methods was sufficient to detect RhD DNA sequences present in concentrations as low as 0.05%. In addition, failure to detect RhD sequences in six cases was not due to low fetal DNA concentrations because the RhD-specific 99-bp PCR fragment was observed in all cases with a gestational age of 21 weeks or less.

Failure to amplify RhD gene sequences in some cases may

be due to genetic variability in the Rh genes.[4] The regions of highest divergence between the RhD and RhCE genes occur in intron 4 and exons 4, 7, and 10.[18] Investigation of the molecular basis of RhD variant phenotypes in different populations has demonstrated gene conversion to be a principal mechanism responsible for polymorphism and gene diversity in the Rh system.[19–21] In addition, gene deletion[4] and point mutations[22] have been identified also. Therefore, to overcome potential misdiagnosis of an RhD-negative fetus, investigators need to further evaluate RhD-negative cases before considering a diagnosis. The use of multiple PCR primers and PCR typing of paternal RhD alleles would improve the sensitivity of this approach in detecting fetal RhD sequences. In cases involving inability to PCR-type paternal RhD alleles, uncertain paternity, or unavailability of the partner, diagnosis of an RhD-negative fetus must not be assumed when a negative result is obtained by the maternal serum DNA test described in this report. The current study reports retrospective analysis of frozen serum specimens in which RhD sequences were detected correctly in 70% of cases. Failure to amplify the RhD sequences in the remaining 30% of cases could have been due to lack of fetal DNA template or to a polymorphism in the paternal RhD allele. Unfortunately, paternal DNA was unavailable for additional testing in these six cases. Clearly, further evaluation of this test to include other primers and larger numbers of cases is necessary before considering this approach for routine clinical use.

There are several advantages to using fluorescent PCR over conventional methods that are relevant to detection of rare fetal sequences in a background of maternal sequences. The use of fluorescent dyes permit visualization of weak signals to reduce experimental time and possibility of contamination. In the current study, detection of RhD fetal sequences by fluorescent PCR required only a single round of amplification. With use of conventional PCR methods, detection of rare fetal RhD sequences in maternal serum DNA required a second round of nested PCR. In addition, the possible risk of contamination increases when aliquots of PCR products are prepared and subjected to additional amplifications. Another advantage of fluorescent PCR is the ability to perform multicolor analysis, enabling amplification of multiple loci of similar size in a single reaction. In the case of Rh gene typing, multiple primers labeled with different fluorescent dyes may be used to amplify simultaneously different sequences within several genes. Combined with the accurate sizing of PCR fragments, multicolor analysis may also eliminate false-positive RhD detection due to background PCR amplification. In fact, we observed that the RhCE-primer pair used in this study generates a background band of 98- to 100-bp size that is indistinguishable from the 99-bp RhD PCR fragment generated by the RhD-primer pair on agarose gels. By using primers labeled with two different colors, as shown in Figure 2, we were able to easily distinguish the RhD- and RhCE-specific PCR products. In the case of the conventional PCR, we confirmed our RhD typing results by independently amplifying the RhD and RhCE gene sequences.

We have demonstrated noninvasive prenatal detection of a single gene disorder by using maternal serum DNA. Although the focus of this study was determination of fetal RhD status, this approach would be applicable to detection of sex-linked disorders, autosomal-dominant disorders of paternal origin, and autosomal-recessive disorders in which both parents have a different mutation. Prospective studies to allow for additional cases and use of fresh serum specimens combined with other PCR primer pairs are necessary to address the feasibility of this test for routine prenatal clinical use.

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Genetic analysis of human DNA recovered from minute amounts of serum or plasma

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Abstract

A rapid and inexpensive method is described where a small amount of serum or plasma was used as the source of DNA for genetic analysis. Using a silica gel matrix DNA was isolated from 50 μl of archived serum or plasma. The specimens were collected from 13 individuals at two separate time points 3–6 years apart. The polymorphic region of second exon of the MHC class II gene HLA DQA1 was amplified using the polymerase chain reaction (PCR) to sufficient quantities to permit genetic analysis using allele-specific oligonucleotides (ASO). Allelic typing of each specimen was performed and the reproducibility of the method was demonstrated in that in all 13 cases the two independently isolated specimens produced the identical ASO binding patterns. No qualitative difference was noted in the amplified product generated from plasma or serum. This study demonstrates (a) that minute amounts of serum or plasma are able to provide sufficient quantity and quality of DNA to permit genetic analyses (b) and that the source of serum can be archived for many years.

Keywords: Genetic analysis; HLA; Polymerase chain reaction; DNA

1. Introduction

The tools of molecular biology have enabled researchers to probe the genomes of plants and animals in such great detail that the identity of an individual (Decorte and Cassiman, 1991; Rogstad, 1993) the evolution of a class of molecules (Gyllensten and Erlich, 1990; Ehmann et al., 1993) or an entire species (Meyer and Dolven, 1992) can be determined with DNA from only a few cells (Erlich and Arnheim, 1992; Arnheim et al., 1990). The current sequencing of the human genome (Watson, 1990; Cantor, 1990) will ensure the number of genes that can be studied will increase greatly. Already systems are available for detailed genetic analyses on the genes of the MHC complex (Gyllensten and Allen, 1991; Wordsworth,

Abbreviations: ASO, allele-specific oligonucleotide; DNA, deoxyribonucleic acid; HLA, human leukocyte antigen; MHC, major histocompatibility complex; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell.

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1991), blood group antigens (Bennett et al., 1993; O'Keefe and Dobrovic, 1993; Corfield et al., 1993), oncogenes (Hruban et al., 1993; Schalken, 1991; Dicker et al., 1990), a variety of genetic diseases (Kaplan et al., 1992; Fong, 1993; McCabe, 1991; Reiss and Cooper, 1990) and many others. However once the genomic loci to be studied are fully characterized, often the most difficult task is obtaining sufficient genetic material to complete the study. The most common source of DNA for genetic analysis is peripheral blood mononuclear cells (PBMC). Although the quantity of DNA isolated from PBMC is usually plentiful (in the tens of μg range) the processing of the cells and subsequent DNA purification do have their drawbacks. Here we present a rapid and inexpensive method of isolating DNA for genetic analysis from serum or plasma. Serum and plasma have the advantages that they can be isolated with minimal equipment requirements under basic laboratory conditions and many laboratories already have large banks of stored serum or plasma from other studies that could be used for genetic analyses. We chose the HLA DQA1 typing system (Erlich and Bugawan, 1990) as the method of genetic analysis because HLA class II typing is ubiquitously used for a variety of purposes including tissue typing for renal transplantation (Papola et al., 1993; Bryan et al., 1993) and the identification of individuals in forensic science (Blake et al., 1992).

2. Materials and methods

2.1. Population sampled and source of biological material

Whole blood was collected from women attending a clinic for sexually transmitted diseases in Nairobi, Kenya who were participating in previously described studies (Ngugi et al., 1988, Simonsen et al., 1990, Plummer et al., 1991). Having given their informed consent, once the nature and possible consequences of the study were explained, whole blood was collected with or without heparin, centrifuged and the plasma or serum, respectively, removed and stored at −70°C. Serum or plasma isolated from 13 individuals in 1985/86 and again in 1990/91 were used in this study.

2.2. DNA isolation

The Bio-Rad Prep-A-Gene DNA purification kit was used for DNA isolation from serum. 50 μl of serum or plasma was diluted with an equal volume of sterile dd$H_2$0 in a 1.5 ml microcentrifuge tube. 300 μl of binding buffer (50 mM Tris, 1 mM EDTA, 6 M $NaClO_4$, pH 7.5) was added to this dilution of serum and mixed. 10 μl of resuspended silica gel matrix was added and incubated for 15 min at room temperature with occasional mixing. After incubation the suspen-

Table 1
Oligonucleotide primers and probes used in HLA DQA1 amplification and genetic typing

Oligonucleotide name	Sequence 5′ → 3′	HLA DQA1 sequences recognized
GH26	GTGCTGCAGGTGTAAACTTGTACCAG	Conserved 5′ region
GH27	CACGGATCCGGTAGCAGCGGTAGAGTTG	Conserved 3′ region
RH83	GAGTTCAGCAAATTTGGAG	1 allele
GH88	CGTAGAACTCCTCATCTCC	1.1 allele
GH76	GTCTCCTTCCTCTCCAG	All but 1.3
GH89	GATGAGCAGTTCTACGTGG	1.2, 1.3, 4 alleles
GH77	CTGGAGAAGAAGGAGAC	1.3 allele
RH71	TTCCACAGACTTAGATTTGAC	2 allele
GH67	TTCCGCAGATTTAGAAGAT	3 allele
GH66	TGTTTGCCTGTTCTCAGAC	4 allele
HE46	CATCGCTGTGACAAAACAT	4.2, 4.3 alleles

sion was centrifuged for 10 s to pellet the silica matrix. The matrix was washed twice in binding buffer and three times in wash buffer (20 mM Tris, 2 mM EDTA, 0.4 mM NaCl, pH 7.4 in 50% ethanol) by resuspending the pellet in 500 μl of the appropriate buffer and centrifuging for 10 s to pellet. The DNA was eluted from the matrix by resuspending the silica matrix in 20 μl of sterile ddH_2O and incubating at 50°C for 15 min. The sample was then centrifuged for 30 s and the DNA containing supernatant was removed and saved. The elution step was repeated once and the supernatants pooled.

2.3. Amplification of the cellular target

The cellular target chosen for amplification was the polymorphic region of the second exon of the MHC class II gene HLA DQA1. Primers and probes were described previously (Erlich and Bugawan, 1990) and are summarized in Table 1. For each sample a cocktail was prepared that contained 25 pmol of each primer (GH26 and GH27), 2.5 U of *Taq* polymerase (Perkin Elmer Cetus), 0.2 mM of each dNTP, 10 μl of 10× buffer (10 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), and H_2O up

Table 2
Binding patterns of HLA DQA1 allele specific oligonucleotides to amplification products of DNA isolated from plasma and serum

Study number	Sample number	Isolation date (D/M/Y)	Binding ASO	HLA DQA1 alleles
1025	1025a	28/6/88	GH66, RH83, GH88, GH89, HE46	1.1; 4.2/4.3
1025	1025b	2/7/91	GH66, RH83, GH88, GH89, HE46	1.1; 4.2/4.3
114	114a	21/2/85	RH83, GH89	1.2/1.3
114	114b	26/4/88	RH83, GH89	1.2/1.3
546	546a	14/11/85	RH83, GH89	1.2/1.3
546	546b	20/3/91	RH83, GH89	1.2/1.3
767	767a	13/1/87	GH67, RH71	2; 3
767	767b	8/1/91	GH67, RH71	2; 3
768	768a	9/1/87	RH83, GH88, RH71, HE46	1.1; 2; 4.2/4.3
768	768b	25/1/91	RH83, GH88, RH71, HE46	1.1; 2; 4.2/4.3
825	825a	24/2/87	GH66, GH89, RH71	2; 4.1
825	825b	22/11/90	GH66, GH89, RH71	2; 4.1
857	857a	6/10/87	RH83, GH89, RH71	1.2/1.3; 2
857	857b	26/3/91	RH83, GH89, RH71	1.2/1.3; 2
870	870a	30/6/87	GH66, RH83, GH88, HE46	1.1; 4.2/4.3
870	870b	22/11/90	GH66, RH83, GH88, HE46	1.1; 4.2/4.3
893	893a	7/8/87	GH66, GH89	4.1
893	893b	20/11/90	GH66, GH89	4.1
896	896a	11/9/87	GH66, GH89	4.1
896	896b	21/1/91	GH66, GH89	4.1
923	923a	29/9/87	GH66, GH89	4.1
923	923b	9/7/91	GH66, GH89	4.1
935	935a	8/9/87	GH66, RH83, GH89	1.2/1.3; 4.1
935	936b	18/7/91	GH66, RH83, GH89	1.2/1.3; 4.1
968	968a	18/2/88	RH83, GH88, GH89, HE46	1.1; 4.2/4.3
968	968b	9/11/90	RH83, GH88, GH89, HE46	1.1; 4.2/4.3
DNA-H_2O	DNA-H_2O			–

DNA was extracted from the samples listed then HLA DQA1 amplified and ASO typed as described in the materials and methods section. This is a summary of the ASO binding patterns and inferred HLA DQA1 allele types from the listed samples. The entire DNA extraction, HLA DQA1 amplification, and ASO typing process was performed on a sample of water (DNA-H_2O) to ensure that no cross-contamination was occurring at any point in the procedure.

to 60 μl. 40 μl of sample template was added to the cocktail and mineral oil was overlaid to prevent evaporation. The amplification protocol consisted of denaturation at 94°C, annealing at 50°C, and extension at 72°C, each step for 30 s, repeated for 30 cycles and ended by 7 min at 72°C to complete extension.

2.4. Product analysis

To ensure amplification of the HLA DQA1 gene was successful 20 μl of the post-amplification reaction mixture was size separated on a ethidium bromide stained 3.5% agarose gel where the quantity and size of the amplification products were verified. The molecular weight standards ran on the gel were *Hae*III digested pBR322 (Boehringer Mannheim). Replicate blots were prepared and each probed with a different ^{32}P-labelled allele-specific oligonucleotide (ASO). Hybridizations were performed using standard conditions (Sambrook et al., 1989). The hybridization temperatures for probes GH66, RH83, GH88, and GH89 was 50°C and 47°C for probes GH67, RH71, GH77, and HE46 (see Table 1 for oligonucleotide sequences). Blots were washed at the specified hybridization temperatures with 1 × SSC (sodium chloride 16.7 mM and sodium citrate 15 mM) and 1% SDS (sodium dodecylsulfate) for 10 min followed by two 30 min washes. The blots were then exposed to X ray film (Kodak X-O-Mat) for 2–24 h with intensifying screens at −70°C and developed.

3. Results

Serum and plasma collected from 13 individuals were used for genetic analysis with the HLA DQA1 typing system. Serum was collected from specimens isolated in 1985–88 and plasma was collected from specimens isolated in 1990 and 1991. Fig. 1 depicts amplification products of the HLA DQA1 amplification system visualized on an ethidium bromide stained 3.5% agarose gel. The predicted length of the HLA DQA1 amplification product is 242 base pairs. Fig. 2 is a representative autoradiogram of replicate southern blots of the HLA DQA1 amplification products probed with ^{32}P-labelled ASOs as described in the methods section. The blot was probed with radiolabelled oligonucleotide HE46 which binds to allele 4 subtypes 4.2 and 4.3. Samples 1025a, 1025b, 768a, 768b, 870a and 870b hybridized with probe HE46. The results of all of the ASO hybridizations are summarized in Table 2. The hybridization temperature used for ASO GH77 (47°C) was not sufficient to differentiate alleles

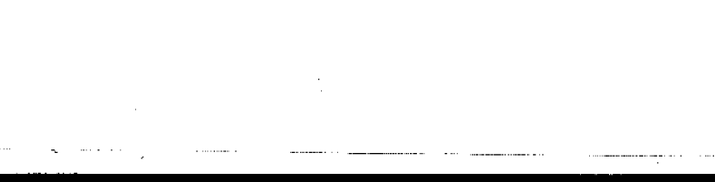

Fig. 1. Amplification of HLA DQA1 locus using DNA isolated from serum or plasma. Amplification products were resolved on an ethidium bromide stained 3.5% agarose gel. To prevent inter-well leakage and to increase spacing between samples while maximizing gel space the gel was loaded in a staggered way. The 'A' series of samples and molecular weight standards were loaded into every second well and electrophoresis was begun. After 15 min the electrophoresis was stopped, the 'B' series of samples, including the molecular weight standards, were loaded into the empty alternate wells and electrophoresis was resumed until completion. The predicted length of the amplification product of HLA DQA1 locus is 242 base pairs. The amplification product for the 'A' series is 242 base pairs when compared to the 'A' molecular weight standard and similarly with the 'B' series of amplification products when compared to the 'B' molecular weight. Only the 'A' molecular weight standards are labelled (in base pairs).

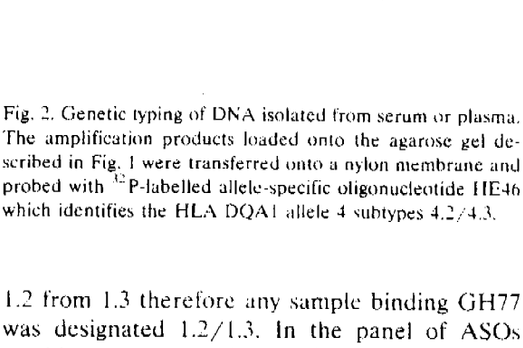

Fig. 2. Genetic typing of DNA isolated from serum or plasma. The amplification products loaded onto the agarose gel described in Fig. 1 were transferred onto a nylon membrane and probed with ^{32}P-labelled allele-specific oligonucleotide HE46 which identifies the HLA DQA1 allele 4 subtypes 4.2/4.3.

1.2 from 1.3 therefore any sample binding GH77 was designated 1.2/1.3. In the panel of ASOs used it was not possible to differentiate alleles 4.2 from 4.3 so any sample binding to GH66 (binds all subtypes of allele 4) but not HE46 (binds subtypes 4.2 and 4.3) was designated as a 4.1 allele and any sample binding both GH66 and HE46 was designated 4.2/4.3.

4. Discussion

The present study has demonstrated that minute amounts of serum or plasma can be used a source of amplifiable DNA. The DNA from 13 individuals was extracted from paired samples of serum or plasma. Fig. 1 illustrates that DNA from each serum or plasma sample was of sufficient quality and quantity to permit amplification of the polymorphic region of the HLA DQA1 locus. Serum was collected in 1985–1988 and plasma was collected in 1990 and 1991. Fig. 1 shows there is no qualitative difference in the amplification products generated from serum ('A' samples) or plasma ('B' samples) therefore both can be used as sources of DNA for genetic analysis.

To illustrate the reproducibility and utility of this method and to demonstrate that the amplification products generated were not a product of contamination from a single source, the amplification products were typed using oligonucleotide probes specific for the alleles of the HLA DQA1 locus (see Table 1). Table 2 illustrates that for each individual, specimens isolated several years apart generated the same ASO binding pattern and therefore demonstrates the reproducibility and validity of this method. Each specimen contains DNA from the maternal and paternal alleles of the HLA DQA1 locus. For that reason we expect the ASOs to define only one allele if homozygous and two alleles if heterozygous. However, samples 768a and 768b bind to three allele-specific oligonucleotides and further analysis is needed to determine if this represents a novel HLA DQA1 allele. The successful use of archived sera for genetic analysis was demonstrated by the generation and subsequent genetic typing of the specific 242 bp HLA DQA1 amplification products from samples 114a and 546a which were isolated in 1985 and have been stored frozen since then. Analysis of larger amplification products generated from archived specimens would have to be assessed on an individual basis.

PBMC are often used as the source of material for genetic analyses. However the use of serum or plasma as the source of genetic material has many advantages over PBMC. The extraction of DNA or RNA from PBMC involves the use of multiple centrifugation steps, the use of caustic organic compounds, it can be time consuming (approximately 4 h from blood to purified DNA) and must be performed within hours of sample collection. In large population based genetic studies that involve hundreds of test individuals often thousand of miles from a fully equipped research laboratory these can be serious drawbacks.

Martin et al. (1992) have described a procedure for the isolation of DNA from serum. Their procedure, however, uses up to 2000 μl of serum or plasma, is very time consuming ($>$ 24 h) and requires the use of an expensive ultracentrifuge. The DNA isolation method described here uses only 50 μl serum or plasma, is rapid (less than 60 min), inexpensive (less than USD 0.50 per test) and has minimal equipment requirements (a microcentrifuge and a water bath). These represent significant advantages over both the extraction of

DNA from PBMC and the method of Martin et al. (1992).

In cases where the identity of paired samples of serum or plasma is in question this method is well suited to determining if they originate from the same individual. This method would prove useful to laboratories which have large banks of archived serum or plasma. This eliminates the need of collecting new PBMC specimens, which can be expensive and logistically difficult. Since this method uses only 50 μl per isolation it ensures that enough serum or plasma remains for other genetic analyses or continued storage.

In conclusion this DNA isolation procedure is suited for even the most basic of laboratories and budgets and could find applications in a broad range of fields including population genetics, organ transplantation and forensic science.

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AB - BACKGROUND: The potential use of plasma and serum for molecular diagnosis has generated interest. Tumour DNA has been found in 'the plasma and serum of cancer patients, and molecular analysis has been done on this material. We investigated the equivalent condition in pregnancy-that is, whether fetal DNA is present in maternal plasma and serum. METHODS: We used a rapid-boiling method to extract DNA from plasma and serum. DNA from plasma, serum, and nucleated blood cells from 43 pregnant women underwent a sensitive Y-PCR assay to detect circulating male fetal DNA from women bearing male fetuses. FINDINGS: Fetus-derived Y sequences were detected in 24 (80%) of the 30 maternal plasma samples, and in 21 (70%) of the 30 maternal serum samples, from women bearing male fetuses. These results were obtained with only 10 microL of the samples. When DNA from nucleated blood cells extracted from a similar volume of blood was used, only five (17%) of the 30 samples gave a positive Y signal. None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had positive results for plasma, serum or nucleated blood cells. INTERPRETATION: Our finding of circulating fetal DNA in maternal plasma may have implications for non-invasive prenatal diagnosis, and for improving our understanding of the fetomaternal relationship.

THE LANCET

Early report

Presence of fetal DNA in maternal plasma and serum

Y M Dennis Lo, Noemi Corbetta, Paul F Chamberlain, Vik Rai, Ian L Sargent, Christopher W G Redman, James S Wainscoat

Summary

Background The potential use of plasma and serum for molecular diagnosis has generated interest. Tumour DNA has been found in the plasma and serum of cancer patients, and molecular analysis has been done on this material. We investigated the equivalent condition in pregnancy—that is, whether fetal DNA is present in maternal plasma and serum.

Methods We used a rapid-boiling method to extract DNA from plasma and serum. DNA from plasma, serum, and nucleated blood cells from 43 pregnant women underwent a sensitive Y-PCR assay to detect circulating male fetal DNA from women bearing male fetuses.

Findings Fetus-derived Y sequences were detected in 24 (80%) of the 30 maternal plasma samples, and in 21 (70%) of the 30 maternal serum samples, from women bearing male fetuses. These results were obtained with only 10 µL of the samples. When DNA from nucleated blood cells extracted from a similar volume of blood was used, only five (17%) of the 30 samples gave a positive Y signal. None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had positive results for plasma, serum or nucleated blood cells.

Interpretation Our finding of circulating fetal DNA in maternal plasma may have implications for non-invasive prenatal diagnosis, and for improving our understanding of the fetomaternal relationship.

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Introduction

The passage of nucleated cells between mother and fetus is well recognised.[1,2] One important clinical application is the use of fetal cells in maternal blood for non-invasive prenatal diagnosis.[3] This approach avoids the risks associated with conventional invasive techniques, such as amniocentesis and chorionic-villus sampling. Substantial advances have been made in the enrichment and isolation of fetal cells for analysis.[3,4] However, most techniques are time-consuming or require expensive equipment.

There has been much interest in the use of DNA derived from plasma or serum for molecular diagnosis.[5] In particular, there have been reports that tumour DNA can be detected by molecular techniques in the plasma or serum of cancer patients.[6–8] Such reports prompted us to investigate whether fetal DNA can be detected in maternal plasma and serum.

Methods

Patients

Pregnant women attending the John Radcliffe Hospital (Oxford, UK) were recruited before amniocentesis or delivery. We obtained approval of the project from the Central Oxfordshire Research Ethics Committee. Informed consent was obtained in each case. 5–10 mL maternal peripheral blood was collected into one tube containing edetic acid and one plain tube. For women undergoing amniocentesis, maternal blood was always taken before the procedure, and 10 mL amniotic fluid was also collected for fetal sex determination. For women recruited just before delivery, fetal sex was noted at the time of delivery. Control blood samples were also taken from ten non-pregnant women, and the samples were processed in the same way as those obtained from the pregnant women.

Sample preparation

Maternal blood samples were processed 1–3 h after venesection. Blood samples were centrifuged at 3000 *g*, and plasma and serum were carefully removed from the edetic-acid-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. After removal of the plasma samples, the red-cell pellet and buffy coat were saved for DNA extraction with a Nucleon DNA extraction kit (Scotlabs, Strathclyde, Scotland, UK). The plasma and serum samples then underwent a second centrifugation at 3000 *g*, and the recentrifuged plasma and serum samples were collected into fresh polypropylene tubes. The samples were stored at −20°C until further processing.

DNA extraction from plasma and serum samples

Plasma and serum samples were processed for PCR by a modified version of Emanuel and Peska's method.[9] 200 µL plasma or serum in a 0·5 mL eppendorf tube was heated at 99°C for 5 min on a heat block. The heated sample was then centrifuged at maximum speed in a microcentrifuge, after which the clear supernatant was collected and 10 µL used for PCR.

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DNA extraction from amniotic fluid

The amniotic-fluid samples were processed for PCR by the method of Rebello and colleagues.[10] 100 μL amniotic fluid was transferred into a 0·5 mL eppendorf tube, and mixed with an equal volume of 10% Chelex-100 (Bio-Rad). After addition of 20 μL mineral oil to prevent evaporation, the tube was incubated at 56°C for 30 min on a heat block. The tube was then vortexed briefly and incubated at 99°C for 20 min. The treated amniotic fluid was stored at 4°C until PCR, and 10 μL was used in a 100 μL reaction.

PCR

The PCR was carried out broadly as described elsewhere[11] with reagents from a GeneAmp DNA Amplification Kit (Perkin Elmer, Foster City, CA, USA). The detection of Y-specific fetal sequence from maternal plasma, serum, and cellular DNA was done as described with primers Y1·7 and Y1·8, designed to amplify a single-copy sequence (DYS14).[12] The Y-specific product was 198 bp. 60 cycles of Hot Start PCR with Ampliwax technology were used on 10 μL maternal plasma or serum, or on 100 ng maternal nucleated blood-cell DNA; each cycle consists of a denaturation step at 94°C for 1 min, and a combined reannealing/extension step at 57°C for 1 min. 40 cycles were used for amplification of amniotic fluid. PCR products were analysed by agarose-gel electrophoresis and ethidium-bromide staining. PCR results were scored before fetal sex was revealed to the investigator.

Results

Serial dilutions of male genomic DNA in 1 μg female genomic DNA were carried out and amplified by the Y-PCR system with 60 cycles of amplification. Positive signals were detected up to the 1/100 000 dilution—ie, the approximate equivalent of a single male cell.

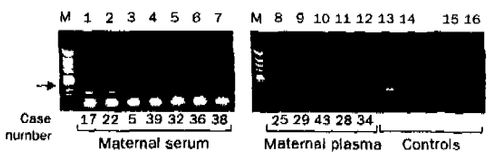
The optimum volume of heated plasma and serum samples for PCR amplification was assessed by subjecting 1 μL, 2 μL, 10 μL, 30 μL, and 50 μL heated plasma or serum samples from male individuals to Y-PCR. The best signal was obtained from 10 μL plasma or serum. Complete inhibition of amplification was reached with 50 μL. Thus, 10 μL heated plasma or serum samples were used for subsequent experiments.

Maternal plasma and serum samples were collected from 43 women who were between 12 and 40 weeks pregnant. There were 30 male and 13 female fetuses. Among the 30 women bearing male fetuses, Y-positive signals were detected in 24 plasma samples and 21 serum samples when 10 μL of the samples was used for PCR (figure and table). When DNA from nucleated blood-cells was used for Y-PCR, positive signals were detected in only five of the 30 cases (table). None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had a positive Y signal when plasma, serum, or cellular DNA was amplified.

Discussion

Our results show that fetal DNA is present in maternal plasma and serum. Use of maternal plasma or serum for the detection of fetal DNA for non-invasive prenatal diagnosis may therefore be possible. Ironically, plasma is the material routinely discarded in the early stages of many DNA-extraction protocols, and also after the density-centifugaton step used by many investigators for non-invasive prenatal diagnosis. This is probably one of the reasons why the presence of fetal DNA in maternal plasma has not been explored previously.

Maternal serum is being used by many centres for biochemical screening of chromosomal aneuploidies and neural-tube defects. An approach by which DNA-based diagnosis is done on serum samples could be incorporated into existing screening programmes.



Amplification of fetal Y-chromosomal sequences from maternal plasma and serum

Lanes 13 and 14=Y-PCR on male genomic DNA (positive controls); lane 13 DNA equivalent to ten male cells; lane 14 DNA equivalent of one male cell. Land 15, 1 μg female genomic DNA (negative control). Lane 16, water (negative control). Arrow marks position of 198 bp Y-PCR product. M=molecular weight marker (ϕX174 DNA digested with *HincII*).

Our data show that fetal DNA can be detected in as little as 10 μL maternal plasma and serum. The detection rate is much higher than that for DNA from nucleated blood cells extracted from a similar volume of whole blood. This finding suggests a relative enrichment of fetal DNA in maternal plasma and serum, a phenomenon analogous to the relative enrichment of tumour DNA in the plasma and serum of cancer patients.[7,8] The low detection rate of fetal DNA sequences for DNA from nucleated blood cells

Case number	Gestation (weeks)	Y-PCR		
		Plasma	Serum	Blood
Male fetuses				
1	12	−		
2	14	+	−	−
3	15	−	+	+
4	15	−	+	−
5	15	+	+	−
6	15	+	+	−
7	15	+	−	−
8	15	+	−	−
9	15	−	−	−
10	15	−	−	−
11	15	−	−	−
12	15	−	−	−
13	16	+	+	+
14	16	+	+	−
15	16	+	+	−
16	16	+	+	−
17	16	+	+	−
18	16	+	+	−
19	16	+	+	−
20	17	+	+	−
21	17	+	+	−
22	22	−	+	−
23	40	+	+	+
24	40	+	+	+
25	40	+	+	+
26	40	+	+	−
27	40	+	+	−
28	40	+	+	−
29	40	−	+	−
30	40	−	−	−
Female fetuses				
31	15	−	−	−
32	16	−	−	−
33	16	−	−	−
34	16	−	−	−
35	16	−	−	−
36	16	−	−	−
37	17	−	−	−
38	17	−	−	−
39	17	−	−	−
40	18	−	−	−
41	40	−	−	−
42	40	−	−	−
43	40	−	−	−

Amplification of fetal Y-chromosomal sequences from maternal plasma, serum, and blood

resulted from the use of only 100 ng DNA, compared with 1 μg in our previous study.[12] We chose 100 ng because this was the average quantity of DNA extracted from 10 μL whole blood with our genomic DNA-extraction method. This approach allows us to compare the relative detectability of fetal DNA in 10 μL plasma or serum and the cellular component of 10 μL whole blood.

The detection rate of fetal DNA in 10 μL plasma and serum is already high at 80% and 70%, respectively, but these rates can probably be improved. 1 mL maternal blood or serum, for example, will result in a one hundred-fold increase in the absolute amount of fetal genetic material available for analysis. Such a magnitude of increase should lead to a robust and non-invasive system for detection of paternally inherited fetal DNA sequences. However, more work is needed before this can be achieved, since the boiling method we used resulted in a relatively impure DNA extract, which inhibited PCR when volumes much larger than 10 μL were used. We are investigating other DNA-extraction methods for plasma and serum. We believe that concentration methods for plasma and serum DNA could potentially be easier than many fetal-cell isolation methods, such as cell sorting and micromanipulation.

The relative merit of the use of plasma or serum samples requires further investigation. In seven cases—2, 7, 8, 9, 10, 22, and 29—there was discordance in the PCR results obtained from the plasma and serum. A possible explanation for the discrepancies is that the quantity of fetal DNA in these cases may have a limiting effect, and thus sampling errors could be contributing to the observed results. Future research with quantitative PCR assays may elucidate this effect.

In four cases with male fetuses, both plasma and serum were negative for fetal DNA (cases 1, 11, 12, and 30). Three of these women were tested at 15 weeks of pregnancy or earlier (cases 1, 11, and 12). Furthermore, of the seven cases in which there was discordance between plasma and serum samples, all but one were tested before 23 weeks. Taken together, these results suggest that the concentration of fetal DNA increases as gestation progresses, possibly owing to the increase in fetal size. This result is analogous to that of Nawroz and colleagues,[8] who detected in head and neck cancer patients mutant plasma DNA predominantly in those with high tumour load. Future studies should investigate the temporal relation between gestation and the appearance and concentration of fetal DNA in maternal plasma.

As well as sex-linked disorders, techniques for fetal-DNA detection in maternal plasma or serum can also be used to detect many paternally inherited DNA sequences that differ from their maternal counterparts. Clinical examples include fetal rhesus D status assessment[13] and detection of certain haemoglobinopathies.[14] The plasma or serum-based approach might also be applicable to screening for chromosomal aneuploidies (such as Down's syndrome) if there is a quantitative difference in the concentration of fetal DNA in maternal plasma and serum between affected and normal pregnancies; this is a situation analogous to the high concentration of fetal cells detectable in pregnancies that involve aneuploid fetuses.[15] Fetal-cell isolation, however, will nevertheless be necessary for the definitive cytogenetic diagnosis of fetal chromosomal aneuploidies, and for the direct mutational analysis of autosomal recessive disorders caused by a single mutation.

The presence of fetal DNA in maternal plasma and serum has not been previously described. The underlying processes that cause free fetal DNA to be released into the maternal circulation have yet to be explained. Possible mechanisms include cell lysis resulting from physical and immunological damage, and developmentally regulated apoptosis of certain fetal tissues. There are interesting similarities between a growing fetus and a neoplasm: both are immunologically foreign to—and have an extensive vascular interface with—their hosts. Investigation of the variations in fetal-DNA concentrations in conditions in which the placental interface is damaged (eg, pre-eclampsia) would be of clinical and biological interest.

Contributors

Y M Dennis Lo and James S Wainscoat initiated the project. Y M Dennis Lo reviewed literature, supervised the daily practical work and analysis of laboratory results. Noemi Corbetta designed the experimental methods and was the main laboratory worker. Paul F Chamberlain, Vik Rai, Ian L Sargent and Christopher W G Redman designed the clinical module of the project. Paul F Chamberlain and Vik Rai were responsible for the clinical correlation of laboratory results. Ian L Sargent, Christopher W G Redman and James S Wainscoat analysed laboratory results. James S Wainscoat designed the DNA-extraction methods, and supervised the writing of the manuscript. All the authors read and contributed to the writing of the paper.

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EXTRACELLULAR DNA IN THE BLOOD OF PREGNANT WOMEN

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An increase has been demonstrated in the concentration of extracellular DNA in the blood of pregnant women, with this increase being most strongly manifested in association with gestosis, a situation which may be linked to the death of decidual cells, with this death taking place by mechanisms of apoptosis. Using the polymerase chain reaction, DNA containing unfragmented Alu repeats has been detected in the blood serum of women in the 1st and 3rd trimesters of pregnancy. Furthermore, when the primer Tc65 was used, fragments of DNA which were flanked by inverted Alu repeats (inter-Alu repeats) were only detected in the blood serum of women in the 1st trimester of pregnancy. The possibilities that the inter-Alu repeats are excreted by cells of the foetus (trophoblast cells) and of the uterus (endometrial cells and lymphocytes) and that such DNA may have a regulatory function in the early stages of pregnancy are discussed.

It has been shown that extracellular DNA is present in human and animal blood (Stroun et al., 1977; Federov, Yaneva, 1982; Valdimirov et al., 1992). An increase in the concentration of extracellular DNA in human blood has been described in association with pathological processes taking place in different tissues of the organism, in particular in association with some inflammatory processes of the gastrointestinal tract, in association with neoplastic and infectious diseases of viral aetiology, and in association with disseminated lupus (Anker et al., 1975; Leon et al., 1977; Shapiro et al., 1983; Stroun et al., 1987; Goto et al., 1991).

The mol. mass of the blood extracellular DNA is on average from 1×10^6 to 15×10^6 Da (Stroun et al., 1977; Federov, Yaneva, 1982). EM microscopy findings indicate that the extracellular DNA in the blood of healthy subjects is double-stranded and linear (Dennin, 1979). According to the findings of Basyukhina and coauthors (1991), human extracellular DNA contains unique sequences. It has also been shown that, in association with disseminated lupus, sequences capable of forming Z-DNA are present in the blood extracellular DNA (Van Helder, 1985).

It is thought that the high molecular weight component of blood extracellular DNA is derived from living cells (Stroun et al., 1977). It has been demonstrated in vitro that some types of cells, especially lymphocytes, excrete high molecular weight DNA into the medium (Rogers et al., 1972; Rogers, 1976; Stroun et al., 1977; Federov, Yaneva, 1982). Furthermore, low molecular weight DNA, corresponding on the basis of mobility to nucleosomes, is also present in the blood. Its content in rat blood increases after total irradiation with X-rays (Belokhvostov et al., 1987; Vladimirov et al., 1992; Tishchenko et al., 1993). According to the findings of Tishchenko and coauthors (1993), the low molecular weight DNA which appears in rat blood after total irradiation with X-rays is enriched in GC sequences. The view has been expressed that the low molecular weight blood DNA is a product of the increased extrachromosomal synthesis of circular DNA which has been detected in many types of human and mammalian cells (Vladimirov et al., 1992). Our attention was attracted by the hypothesis that the increase in the level of extracellular low molecular weight DNA in rat blood following irradiation is a consequence of an increase in the activity of Ca/Mg endonuclease in the cells (Tishchenko et al., 1993). This hypothesis accords very well with findings of an increase in the processes of apoptosis in the cells of different tissues of the organism following irradiation (Hanson, Komar, 1985).

From what has been said it is clear that analysis of the extracellular DNA in human and animal blood is of both theoretical and practical interest. The possibility is not excluded that the nucleotide composition of the blood extracellular DNA is not so random that the features of the processes of differentiation and cell death, which are taking place in different tissues at every single moment of the life of the organism, are not reflected in it. All this determined the choice of the blood of pregnant women as the object of our present investigation. According to available information, cell proliferation and differentiation, and cell death, all take place in the uterus in association with pregnancy (Federov, Kalashnikova, 1986; Mikhailov et al., 1989, 1992a, 1992b; Mikhailov, 1993). It was anticipated that these processes would exert an influence on the specific nature of the nucleotide composition of the extracellular DNA in the blood of pregnant women.

Materials and methods

Blood serum samples from men, non-pregnant women, women in the 1st and 3rd trimesters of pregnancy and women suffering from late toxicosis of pregnancy were investigated. The blood was withdrawn with a syringe from the ulnar vein under sterile conditions and placed in a centrifuge tube; The tube was

then left at room temperature to allow coagulation to take place. Immediately after the thrombus had formed, it was detached from the walls of the tube and centrifugation was carried out at 400 g for 10 min. The serum was centrifuged once again at 2000 g for 10 min and at 4°C. The serum which was obtained in this way was stored at -60°C. The use of serum instead of plasma for analysing the blood DNA can be justified if the stipulations of forming the thrombus at room temperature and immediately separating the serum from the thrombus are observed (Leon et al., 1977; Shapiro et al., 1983). This was in fact done in the present investigation.

The serum was subsequently treated twice with phenol, and with mixtures of phenol and chloroform (1 : 1) and chloroform and isoamyl alcohol (24 : 1), and was precipitated with ethanol at -20°C. The DNA preparations were analysed in a 1% agarose gel and were then used as templates in association with performing a polymerase chain reaction (PCR).

The PCR was performed using thermophilic Thermus thermophilus DNA polymerase. 30-35 cycles of amplification were carried out. The amplified fragments were analysed in 8% PAGE. The temperature for annealing the primers was 55°C for primers B1 and C2 at 60°C for the primer Tc65, and the concentration of Mg^{2+} ions was 2-5 mM. We synthesized the primers for the PCR on a Pharmacia Gene assembler appliance using the solid-phase phosphite triester method in the β-cyanoethyl modification. The sequence of the primer Tc65 was taken from literature sources (Nelson et al., 1989), while the sequences of the remaining primers were found using the Oligo (Microsoft) program from the consensus sequence of Alu repeats of the MF family, and were designated by us as B1 and C2.

When the primer pair B1 and C2 were used to perform the PCR, a 239 base pair Alu repeat was amplified as a result of the reaction. When only the one primer Tc65 was used in the PCR, the amplification resulted in DNA fragments which were flanked by two Alu repeats whose 3' end regions were facing each other (inter-Alu repeats).

The sequences of the primers were as follows:

Tc65-5'-AAGTCGCGGCCGCTTGCAGTGAGCCGAGA-3'

B1-5'-CCTGTAATCCCAGCACTTTGGGAGGC-3'

C2-5'-CCCAGGC T GGAGTGC A GTGG-3'
G G

Results and discussion

In the study, we aimed at making a detailed study of the changes in the concentration of DNA in the blood serum of pregnant women. According to the findings of authors who used radioimmunological methods of analysis (Leon et al., 1977; Shapiro et al., 1983), the concentration of DNA in the blood serum of healthy donors varied from 0 to 0.1 µg/ml; according to the findings of authors who used biochemical methods (Anker et al., 1975; Stroun et al., 1987), the concentration varied from 0.1 to 0.6 µg/ml. Our findings with regard to the concentration of DNA in the blood of men and non-pregnant women were in accord the findings from the literature.

On the basis of the data obtained by us, the concentration of low molecular weight DNA increases, in the first instance, in association with pregnancy, with this increase being most strongly expressed in association with gestosis. This DNA is from 150 to 2500 base pairs in size. The view is this that an increase in the concentration of low molecular weight extracellular DNA in the blood is a consequence of an increase in the synthesis of extrachromosomal DNA (Vladimirov et al., 1992). However, in evaluating the findings which are presented, it is necessary to take into account the fact that a substantial quantity of endometrial cells die during pregnancy, especially at the end of pregnancy and in connection with gestosis (Kottsovia et al., 1989; Mikhailov et al., 1992a). According to our findings, the death of decidual cells is accompanied by the progressive loss of nuclear DNA and the fragmentation of peripheral parts of the nuclei and cytoplasm of cells, and apparently proceeds by way of apoptosis (Mikhailov et al., 1992a, 1992b; Mikhailov, 1993). One of the main apoptosis-type mechanisms of cell death is the internucleosomal degradation of DNA as a consequence of the activation of Ca^{2+} endonuclease (Wyllie, 1980). That which has been said does not exclude the possibility of the extrachromosomal copying of DNA as being the source of the blood extracellular DNA. Regardless of whether the source of the blood extracellular DNA is an increased synthesis of extrachromosomal DNA or the apoptotic death of cells, the presence in this DNA of Alu repeats bears witness to its nuclear origin (Figure 1; see enclosure VIII).

One's attention is drawn to the presence of inter-Alu repeats in the DNA found in the blood serum of pregnant women. It is known that approximately one-third of human Alu repeats are inverted repeats and that the mean distance between the inverted pairs is approximately 50 kbp (Jelinek, Schmid, 1982). The matrix of such inverted repeats evidently contains a unique internal fragment. Several types of inter-Alu repeats exist. Using the primer Tc65, we only detected inter-Alu repeats in the blood of women in the 1st trimester of pregnancy (Fig. 2).

When PCR is carried out using the Tc65 primer, the only DNA fragments amplified are those in which the spacer DNA is flanked with Alu repeats whose 3' regions are mutually facing each other, which makes these fragments even more unique. Thus, if the presence in the blood of pregnant women of an increased quantity of low molecular weight DNA fragments is a manifestation of an increase in cell death taking place in the uterus at all stages of pregnancy, the presence of inter-Alu repeats then points to the operation of other mechanisms for the entry of such sequences into the blood samples. It is important that the inter-Alu repeats were only detected in the blood of women in the 1st trimester of pregnancy. This fact most likely reflects a difference in the content of the cellular processes which are characteristic of the early and late stages of pregnancy. If the death of decidual cells is observed at the end of pregnancy, which explains the increase in the concentration of low molecular weight DNA in the blood, decidualization of the endometrium and growth of the foetal sack, that is the process of the differentiation of uterine mucosal cells and trophoblast cells, then take place in the initial period of pregnancy, when implantation and placentation of the embryo occur in the uterus.

It is probable that excretion of the inter-Alu repeats from the cells takes place at early stages in pregnancy. The question of which type of cell might excrete inter-Alu repeats is of great interest. Investigators are also faced with this question when considering the sources of the high molecular weight DNA in the serum (see review; Federov, Yaneva, 1982). The excretion of DNA in vitro is well characterized in the case of lymphocytes (Federov, Yaneva, 1982). It is suggested that lymphocytes could be a source of such DNA in association with neoplastic diseases. A significant infiltration of lymphocytes is also observed in the uterine mucus in the early stages of pregnancy.

Thus, cells of the foetus (trophoblast) and the maternal organism (endometrial cells and lymphocytes) may excrete DNA in the early stages of human pregnancy. With regard to what has been said above, and also with regard to the transposon-like and recombinant nature of Alu repeats (Tomilin, 1992), it is possible to surmise that the inter-Alu repeats, which we have detected in the blood serum of pregnant women, may perform some type of regulatory function in the early periods of pregnancy. It would be of particular interest to clone and sequence these fragments. What has been said does not exclude the presence in the blood of pregnant women of other inter-Alu repeats which might be revealed using other primers and might have their own peculiarities with regard to their distribution in the blood during pregnancy.

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EXTRACELLULAR DNA OF PREGNANT WOMEN BLOOD

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The level of extracellular DNA increases in the blood of women during pregnancy. By means of PCR, the full-size Alu repeats were observed among extracellular blood. DNA repeats of pregnant women. Furthermore, with Tc65 type primer the PCR method allowed to observe in the blood DNA fragments flanked by inverted Alu repeats (inter Alu repeats). The presence of such a type of inter Alu repeats was estimated in the blood of women being in the first trimester of pregnancy only, but was not estimated among blood DNA fragments of women of the last trimester of pregnancy. It is discussed which types of cells may serve as a source of extracellular blood DNA (either trophoblast cells, lymphocytes, or decidual cells), the significance of such DNA for pregnancy being appreciated.

#7

◆ REVIEW ARTICLE ◆

Fetal RhD genotyping from maternal plasma

Y M Dennis Lo

The prenatal diagnosis of fetal rhesus D (RhD) status is useful for the management of RhD-negative women with partners heterozygous for the *RHD* gene. Conventional methods for prenatal fetal RhD status determination involve invasive procedures such as fetal blood sampling and amniocentesis. The recent demonstration of the existence of cell-free fetal DNA in maternal plasma and serum opens up the possibility of determining fetal RhD status by analysis of maternal plasma or serum DNA. This possibility has recently been realized by three independent groups of investigators. This development represents an important step towards the routine application of noninvasive fetal blood group diagnosis in sensitized pregnancies and may become a model for developing safer noninvasive prenatal tests for other single-gene disorders.

Key words: blood grouping; DNA; polymerase chain reaction; pregnancy; prenatal diagnosis.

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Introduction

The rhesus blood group system is of great importance in transfusion and clinical medicine, being involved in haemolytic disease of the newborn, transfusion reactions and autoimmune haemolytic anaemias (1). Approximately 85% of the white population is rhesus D (RhD)-positive the remaining 15% being RhD-negative (1). If a RhD-negative woman bears a RhD-positive fetus, there is a finite chance that she will become sensitized, and in subsequent pregnancies involving RhD-positive fetuses the passage of maternally produced anti-D antibody across the placenta may result in fetal haemolysis (1). The widespread use of rhesus immunoglobulin prophylaxis in RhD-negative mothers has reduced, but not completely eliminated, the occurrence of maternal sensitization (2). In cases where the RhD-negative pregnant mothers are heavily sensitized, a variety of invasive diagnostic and therapeutic procedures are necessary to help reduce perinatal morbidity and mortality (3).

In a case where the father is heterozygous for RhD (accounting for approximately 56% of RhD-positive white individuals (1)), knowledge of the RhD status of the fetus is important in the clinical management of the case as no further diagnostic or therapeutic procedures are necessary if the fetus is RhD-negative. Prior to the cloning of the *RHD* gene (4), the prenatal determination of fetal RhD status was primarily determined by fetal blood sampling (3) and it was therefore reserved for potentially severely affected cases. The dual risks of pregnancy loss and increasing maternal sensitization impose limits on the timing of fetal blood sampling, which is in any event technically very difficult before 18 weeks of gestation. With the cloning of the *RHD* gene (4), it has become possible to determine fetal RhD status by polymerase chain reaction (PCR) analysis of amniotic fluid (5–8) and chorionic villus samples (5). However, as amniocentesis or chorionic villus sampling are associated with several well-recognized risks (9), including further immunological sensitization of the mother as a result of possible fetomaternal haemorrhage after invasive prenatal diagnosis (10, 11), several groups have investigated the possibility of carrying out noninvasive fetal RhD genotyping.

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Fetal nucleated cells in maternal blood for fetal RhD genotyping

Investigators have long suspected that there is trafficking of nucleated fetal cells into the maternal circulation during pregnancy (12, 13). However, the rarity of these cells in the maternal circulation has delayed the early exploitation of this phenomenon for prenatal diagnosis. With the development of molecular biology, there is significant interest worldwide in the use of fetal nucleated cells in maternal circulation for noninvasive prenatal diagnosis (14). The first demonstration that circulating fetal nucleated cells are detectable in maternal circulation by using DNA-based techniques was achieved in 1989, when Y-chromosomal sequences were detected in the blood cell DNA from pregnant women bearing male fetuses (15). The extension of this technology to prenatal RhD genotyping was achieved soon following the cloning of the *RHD* gene (16–18). However, the rarity of circulating nucleated fetal cells represents a major obstacle to reliable diagnosis. Many investigators have described a variety of approaches to enrich fetal cells from maternal blood, including fluorescence-activated cell sorting (19, 20), magnetic-activated cell sorting (21), micromanipulation (22), charge flow separation (23) and fetal cell culture (24, 25). The use of fetal cell enrichment or isolation techniques has allowed the potential improvement in the diagnostic accuracy of fetal RhD genotyping from maternal blood (26, 27). However, the reliability of the reported systems is still not high enough for routine clinical application.

A number of investigators have explored the possibility of detecting mRNA transcripts of the *RHD* gene as an alternative approach for prenatal fetal RhD genotyping from maternal blood (28, 29). However, the number of cases reported is still relatively small and further evaluation of the diagnostic accuracy of this approach is needed.

Fetal cell-free DNA in maternal plasma

It has been known for many years that cell-free DNA is present in the plasma and serum of humans (30, 31). It is noteworthy that the concentration of cell-free DNA has been demonstrated to be elevated in certain pathological conditions, including cancer (31, 32), systemic lupus erythematosus (33) and pulmonary embolism (34). With modern molecular biological techniques, detailed characterization of the nature of cell-free DNA is possible, and it has been demonstrated that circulating DNA in cancer patients possesses genetic or epigenetic alterations present in the primary tumour (35–37), indicating that at least some of this DNA is liberated by the tumour cells.

The presence of tumour DNA in the circulation of cancer patients has prompted investigators to look for other biological scenarios where nonhost DNA may be found in the circulation. This line of reasoning has inspired our group to search for cell-free fetal DNA in the plasma and serum of pregnant women (38). This work has led to the first demonstration that cell-free fetal DNA is indeed present in the plasma of pregnant women (38). Furthermore, as fetal DNA is detectable in as little as 10 μL of maternal plasma and serum, the relative concentration of fetal DNA can be deduced to be very high (38). This deduction has indeed been confirmed by the use of real-time quantitative PCR, which shows that fetal DNA constitutes approximately 3% of the total maternal plasma DNA during the second trimester, with further increase as gestation progresses (39). It has also been demonstrated that following delivery, cell-free fetal DNA is cleared very rapidly, with a half-life in the order of minutes, from maternal circulation (40).

These results indicate that circulating cell-free DNA may be a valuable source of fetal genetic material for noninvasive prenatal diagnosis. The high relative concentration suggests that expensive and time-consuming fetal DNA enrichment procedures would not be necessary to use maternal plasma fetal DNA for diagnostic purposes. The rapid clearance of cell-free fetal DNA from maternal blood is another desirable feature which suggests that it is extremely unlikely that fetal DNA would persist from one pregnancy into the next, unlike certain fetal nucleated cell populations which have been demonstrated to persist in the mother for years following delivery (41).

Fetal RhD genotyping from maternal plasma and serum

The use of cell-free fetal DNA in maternal plasma and serum for noninvasive fetal RhD genotyping has been achieved independently by three groups (42–44). Our group reported the use of a real-time fluorogenic PCR system towards exon 10 of the *RHD* gene and applied this system to maternal plasma samples collected from 57 pregnant women covering all three trimesters of pregnancy (42). Lo et al were able to obtain reliable fetal RhD status determination from the 15th week of gestation and beyond (42). False-negative results were obtained from a proportion of first trimester samples, possibly because of the low concentration of fetal DNA at this stage of gestation (39). Faas and co-workers studied predominantly second trimester samples by using a PCR system towards exon 7 of the *RHD* gene and were able to obtain reliable fetal RhD determination in all their samples (43). Bischoff and co-workers utilized both conventional PCR and a fluorescent PCR system towards exon 7 of the *RHD*

gene and were able to detect fetal *RHD* sequences in 70% of archival serum samples from women carrying RhD-positive fetuses (44).

The overall message from these studies is that noninvasive fetal RhD genotyping from maternal plasma and serum is possible. It also appears from the studies by our group (42) and Faas et al (43) that reliable diagnosis is possible by using current protocols from the second trimester of pregnancy onwards. However, the three groups of investigators have important differences in their methodologies, which require further discussion. With regard to detection systems, these investigators have reported the use of real-time fluorogenic PCR (42), conventional PCR (43, 44) and fluorescent PCR followed by capillary electrophoresis (44). It is possible that for large-scale application, real-time PCR, with its rapidity and lack of requirement for postamplification manipulation, may be the method of choice. To maximize the robustness of the assays, it is recommended that future detection systems should target multiple exons of the *RHD* gene.

These three groups of investigators used different DNA extraction methods from plasma and serum. Thus, we (42) and Faas et al (43) used an identical column-based DNA purification method (39). Bischoff and co-workers (44), on the other hand, used a boiling method (38) for the purification of serum DNA. As a large proportion of the serum sample coagulates during the boiling process (38), this method does not allow the full utilization of a particular serum sample for diagnostic purposes. Furthermore, as the boiled serum is still a relatively impure mixture, PCR inhibitors have been demonstrated in this type of material (38). These factors might contribute towards the relatively low sensitivity of the system reported by Bischoff and co-workers (44).

Another potentially important difference between these groups of investigators concerns the choice of plasma (42, 43) or serum (44) for analysis. It has been shown that the absolute concentrations of fetal DNA in maternal plasma and serum are comparable (39). However, increased amounts of maternal DNA have been found in serum when compared with plasma, possibly as a result of the liberation of DNA during the clotting process (39). These results indicate that a higher maternal background is present when serum is used. This may be detrimental for the detection of fetal DNA, especially when less sensitive detection methods are used.

Our group and Faas et al used predominantly prospectively collected samples for the studies (42, 43), whereas Bischoff and co-workers (44) reported the use of retrospectively collected and stored serum samples for analysis. Bischoff and co-workers suggested that DNA degradation might occur during the freezing and thawing of the stored serum samples and might contribute in part to their detection efficiency of less than 100% (44). An additional danger associated with archival plasma or serum material might be sample contamination from other laboratory sources (45). These considerations underscore the desirability to use prospectively collected samples processed especially for fetal DNA studies for future investigations.

Future directions

Initial data reporting the feasibility of fetal RhD genotyping from maternal plasma and serum are encouraging. It is likely that future improvements in technology may allow more accurate diagnosis to be made and potentially extend the applicability of this method to the first trimester of pregnancy. Apart from aiding the clinical management of previously sensitized RhD-negative women, noninvasive fetal RhD genotyping may also allow the rationalization of anti-D immunoprophylaxis (46). Thus, provided that the fetal RhD genotype can be determined accurately, it may be possible to restrict anti-D immunoprophylaxis only to RhD-negative women carrying RhD-positive fetuses. In other words, RhD-negative women carrying RhD-negative fetuses, who are not at risk of sensitization, might be spared an unnecessary injection.

A second area which warrants investigation is the relationship between fetal DNA levels in maternal plasma and fetal haemolysis secondary to rhesus incompatibility. The mechanisms underlying the liberation of fetal DNA into maternal blood are incompletely understood (47). However, it is possible that fetal nucleated red cell destruction might result in increased fetal DNA liberation into maternal plasma. If this hypothesis turns out to be correct it is possible that fetal DNA analysis from maternal plasma may also be useful for the noninvasive monitoring of the severity of fetal haemolysis.

The success in the detection of fetal-derived *RHD* gene in the plasma and serum of pregnant women opens up the possibility that a similar approach may be used for other single-gene disorders. This development is potentially possible for the detection of paternally inherited genes, DNA polymorphisms or mutations that are distinguishable from the maternally inherited counterparts (48). The single-gene disorders that are possible candidates for analysis of this type include cystic fibrosis and β-thalassemia. Maternal plasma DNA analysis thus represents another powerful addition to our expanding armamentarium for the early detection of fetal genetic disorders.

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Abstract

Background The ability to determine fetal RhD status noninvasively is useful in the treatment of RhD-sensitized pregnant women whose partners are heterozygous for the *RhD* gene. The recent demonstration of fetal DNA in maternal plasma raises the possibility that fetal RhD genotyping may be possible with the use of maternal plasma.

Methods We studied 57 RhD-negative pregnant women and their singleton fetuses. DNA extracted from maternal plasma was analyzed for the *RhD* gene with a fluorescence-based polymerase-chain-reaction (PCR) test sensitive enough to detect the *RhD* gene in a single cell. Fetal RhD status was determined directly by serologic analysis of cord blood or PCR analysis of amniotic fluid.

Results Among the 57 RhD-negative women, 12 were in their first trimester of pregnancy, 30 were in their second trimester, and 15 were in their third trimester. Thirty-nine fetuses were RhD-positive, and 18 were RhD-negative. In the samples obtained from women in their second or third trimester of pregnancy, the results of RhD PCR analysis of maternal plasma DNA were completely concordant with the results of serologic analysis. Among the maternal plasma samples collected in the first trimester, 2 contained no *RhD* DNA, but the fetuses were RhD-positive; the results in the other 10 samples were concordant (7 were RhD-positive, and 3 RhD-negative).

Conclusions Noninvasive fetal RhD genotyping can be performed rapidly and reliably with the use of maternal plasma beginning in the second trimester of pregnancy. (N Engl J Med 1998;339:1734-8.)

THE Rh blood-group system is involved in hemolytic disease of the newborn, transfusion reactions, and autoimmune hemolytic anemia.[1] Despite the widespread use of Rh immune globulin prophylaxis in RhD-negative pregnant women, Rh isoimmunization still occurs.[2] In cases in which the father is heterozygous for the *RhD* gene, and the mother is RhD-negative, there is a 50 percent chance that the child will be RhD-positive. Prenatal determination of RhD status in these cases is clinically useful because no further testing or therapeutic procedures will be necessary if the fetus is RhD-negative. If the fetus is RhD-positive, further studies will be necessary to determine the level of fetal hemolysis (e.g., by fetal-blood sampling).

The human *RhD* gene[3] has been cloned, and it is absent in RhD-negative subjects.[4] Fetal RhD status has been determined in samples of amniotic fluid and chorionic villi with the use of techniques based on the polymerase chain reaction (PCR).[5] However, because of the invasive means by which such samples are obtained, these approaches increase the risk of further sensitizing the mother. To circumvent this risk, several groups have investigated the possibility of determining fetal RhD status through the use of fetal cells isolated from maternal blood.[6-9] The main problem with this approach is that the procedures needed to isolate sufficient numbers of fetal cells from maternal blood are time consuming, technically demanding, and expensive.[7,8] An alternative approach based on the detection of *RhD* messenger RNA in fetal nucleated red cells has also been described,[10] but the small number of subjects analyzed precludes any firm conclusion as to the reliability of this method.

In a recent study, we identified fetal DNA in maternal plasma and serum.[11] Therefore, in the current study, we assessed the feasibility of fetal RhD genotyping using fetal DNA extracted from plasma samples from RhD-negative pregnant women.

METHODS

Subjects

We collected 10-ml blood samples from 30 blood donors who were positive on serologic testing for RhD and 30 blood donors who were negative at the Department of Hematology, John Radcliffe Hospital, Oxford, United Kingdom. We used these samples to establish the accuracy of the RhD PCR system.

To assess the value of the system for prenatal diagnosis, we collected 10-ml blood samples from 57 women with singleton pregnancies who were patients at the Nuffield Department of Obstetrics and Gynecology, John Radcliffe Hospital. Twelve women were in the first trimester of pregnancy (7 to 14 weeks), 30 were in the second trimester (15 to 23 weeks), and 15 were in the third trimester (37 to 41 weeks). Ten were primigravidas. The blood samples were collected from the women who were in the first trimester of pregnancy during a routine prenatal checkup. Blood

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samples from the women in their second trimester were collected just before routine amniocentesis; 10 ml of amniotic fluid was also collected for fetal RhD genotyping. The blood samples were collected from the women in their third trimester just before delivery. For the women who were studied during the first and third trimesters, a sample of cord blood was collected after delivery for the determination of fetal RhD status by serologic methods. The project was approved by the Central Oxfordshire Research Ethics Committee, and all the women gave informed consent.

Preparation of Samples

The blood samples were collected in tubes containing EDTA and centrifuged at 3000×*g*, and the plasma was then transferred into plain polypropylene tubes, with care taken to ensure that the buffy coat was not disturbed. The buffy coat was then removed and stored at −20°C until further processing. The plasma samples were recentrifuged at 3000×*g*, and the supernatants were stored at −20°C until further processing.

DNA Extraction

DNA was extracted from samples of plasma (800 μl), buffy coat, and amniotic fluid (200 μl each) with a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the "blood and body fluid protocol" recommended by the manufacturer.[12] An elution volume of 50 μl was used for the final washing of the DNA from the column.

Real-Time Fluorogenic PCR Analysis

Real-time fluorogenic PCR analysis was performed with a Perkin–Elmer Sequence Detector (model 7700, Perkin–Elmer Applied Biosystems, Foster City, Calif.), which is a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically.[13] The RhD fluorogenic PCR system consisted of the amplification primers RD-A (5′CCTCTCACTGTTGCCTGCATT3′) and RD-B (5′AGTGCCTGCGCGAACATT3′) and a dual-labeled fluorescent probe, RD-T (5′(FAM)TACGTGAGAAACGGTCATGACAGCAAAGTCT(TAMRA)3′; FAM [6 carboxyfluorescein] and TAMRA [6 carboxytetramethylrhodamine] were the fluorescent reporter dye and quencher dye, respectively).[13] The primers and probe were targeted toward the 3′ untranslated region (exon 10) of the *RhD* gene.[3] The β-globin PCR system consisted of the amplification primers and probe as previously described.[14] The fluorescent probes contained a 3′-blocking phosphate group to prevent extension of the probe during the PCR. Combinations of primers and probes were designed with Primer Express software (Perkin–Elmer). Sequence data for the *RhD* gene were obtained from the GenBank data base (accession number, X63097).

The fluorogenic PCR reactions were set up according to the manufacturer's instructions in a reaction volume of 50 μl with all components except the fluorescent probes and amplification primers obtained from a TaqMan PCR Core Reagent Kit (Perkin–Elmer). The RhD and β-globin fluorescent probes were custom-synthesized by Perkin–Elmer and were used at concentrations of 25 nM and 100 nM, respectively. The PCR primers were synthesized by Life Technologies (Gaithersburg, Md.) and were used at a concentration of 300 nM. A total of 5 μl of the extracted plasma or amniotic fluid DNA was used for amplification; for buffy-coat DNA, 10 ng was used. DNA amplifications were carried out in 96-well reaction plates that were designed to capture optical data (Perkin–Elmer).

Thermal cycling was initiated with a two-minute period of incubation at 50°C to allow time for the enzyme uracil *N*-glycosylase, which destroys any contaminating PCR amplicons, to act. This step was followed by initial denaturation for 10 minutes at 95°C and then by 40 cycles of denaturation at 95°C for 15 seconds and reannealing and extension for 1 minute at 60°C.

Amplification data collected by the Sequence Detector and stored in a Macintosh computer (Apple, Cupertino, Calif.) were analyzed with Sequence Detection System software (Perkin–Elmer). The threshold of detection was set at 10 SD above the mean baseline fluorescence calculated from cycles 1 to 15.[13] An amplification reaction in which the intensity of fluorescence increased above the threshold during the course of thermal cycling was defined as a positive reaction.

Anticontamination Measures

Strict precautions against contamination of the PCR assay were used.[15] Aerosol-resistant pipette tips were used to handle all liquids. Separate areas were used to set up amplification reactions, add DNA template, and carry out amplification reactions. The use of the Sequence Detector offered an extra level of protection in that its optical-detection system obviated the need to reopen the reaction tubes after the completion of the amplification reactions, thus minimizing the possibility of carryover contamination. In addition, the PCR assay included a further anticontamination measure in the form of preamplification treatment with uracil *N*-glycosylase, which destroyed uracil-containing PCR products.[16] Multiple water blanks were included as negative controls in every analysis.

RESULTS

The RhD PCR system was used to genotype buffy-coat DNA extracted from the 30 RhD-positive blood donors and the 30 RhD-negative blood donors. There was complete concordance between the results of RhD PCR genotyping and the serologic results.

To determine the sensitivity of fluorogenic RhD PCR analysis, genomic DNA from an RhD-positive subject was diluted serially both in water and in 1 μg of genomic DNA from an RhD-negative subject. The smaller the amount of DNA, the more amplification cycles were needed to produce detectable amounts of fluorescent reporter molecules (Fig. 1). Positive signals were detected with as little DNA as the approximate amount (7.8 pg) contained in a single RhD-positive cell.

All 57 of the pregnant women were RhD-negative on serologic testing. Analysis of DNA extracted from buffy-coat samples from the 45 women who were in the second or third trimester of pregnancy revealed no *RhD* DNA, a finding in agreement with the serologic results. Among the 57 fetuses, 39 were RhD-positive and 18 were RhD-negative on serologic analysis of cord blood or PCR testing of amniotic fluid.

The results of the RhD PCR assay of plasma samples from the 57 women are shown in Table 1. Representative amplification data are shown in Figure 2. Among the women who were in the second or third trimester of pregnancy, there was complete concordance between results of the fetal RhD genotyping with use of the RhD PCR assay of maternal plasma samples and the results obtained from genotyping of amniotic fluid or serologic testing of cord blood. Plasma samples from two women in the first trimester of pregnancy who were carrying RhD-positive fetuses, with gestational ages of eight and nine weeks, yielded false negative results. The results in the other 10 women in their first trimester of pregnancy were concordant: 7 were RhD-positive on PCR

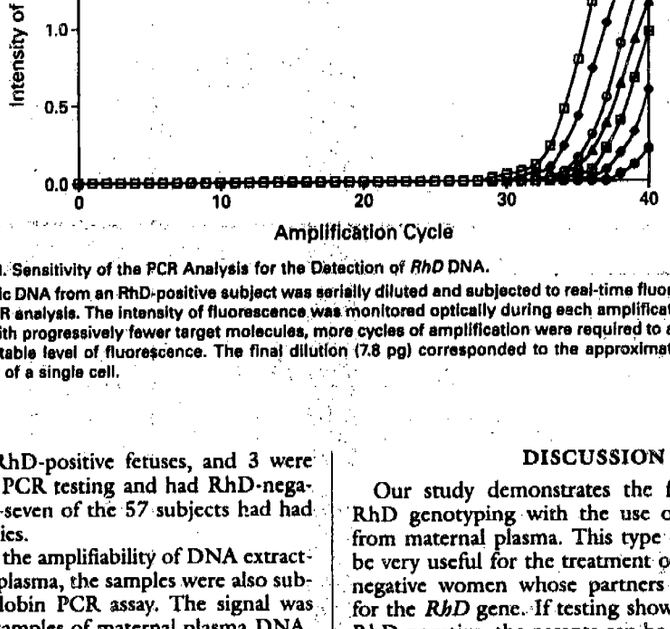

Figure 1. Sensitivity of the PCR Analysis for the Detection of *RhD* DNA.
Genomic DNA from an RhD-positive subject was serially diluted and subjected to real-time fluorogenic RhD PCR analysis. The intensity of fluorescence was monitored optically during each amplification cycle.[13] With progressively fewer target molecules, more cycles of amplification were required to achieve a detectable level of fluorescence. The final dilution (7.8 pg) corresponded to the approximate DNA content of a single cell.

testing and had RhD-positive fetuses, and 3 were RhD-negative on PCR testing and had RhD-negative fetuses. Forty-seven of the 57 subjects had had previous pregnancies.

As a control for the amplifiability of DNA extracted from maternal plasma, the samples were also subjected to the β-globin PCR assay. The signal was positive in all 57 samples of maternal plasma DNA.

Table 1. Results of RhD Genotyping of Fetuses of RhD-Negative Women with the Use of the RhD PCR Assay.*

Trimester of Pregnancy	RhD-Positive Fetus†	RhD-Negative Fetus†
	no. of positive fetuses on PCR testing/total no. of fetuses (%)	
First	7/9 (78)	0/3
Second	22/22 (100)	0/8
Third	8/8 (100)	0/7

*The RhD PCR assay used plasma samples from the women.

†The RhD status was determined by serologic analysis of cord-blood samples in the case of samples obtained during the first or third trimester and by PCR testing of amniotic fluid in the case of samples obtained during the second trimester.

DISCUSSION

Our study demonstrates the feasibility of fetal RhD genotyping with the use of DNA extracted from maternal plasma. This type of analysis should be very useful for the treatment of sensitized RhD-negative women whose partners are heterozygous for the *RhD* gene. If testing shows that the fetus is RhD-negative, the parents can be reassured that the fetus is not at risk. On the other hand, if testing shows that the fetus is RhD-positive, treatment can be planned. The advantage of this test, which analyzes maternal plasma, is that neither the mother nor the fetus is exposed to the risks normally associated with amniocentesis or chorionic-villus sampling.[17] An additional important advantage of this approach is the avoidance of further immunologic sensitization as a result of fetomaternal hemorrhage after invasive procedures.[18,19]

Our data suggest that the results of the RhD PCR test are reliable beginning in the second trimester. The availability of such early, reliable results gives clinicians sufficient time to plan for further tests or treatment such as fetal-blood sampling and fetal transfusion,[20,21] which are usually performed beginning in the middle of the second trimester. The results for two first-trimester samples were false negative, presumably because of the low concentration of fetal DNA in maternal plasma at that time.[14]

This test may also have an application in the routine testing of nonsensitized RhD-negative pregnant

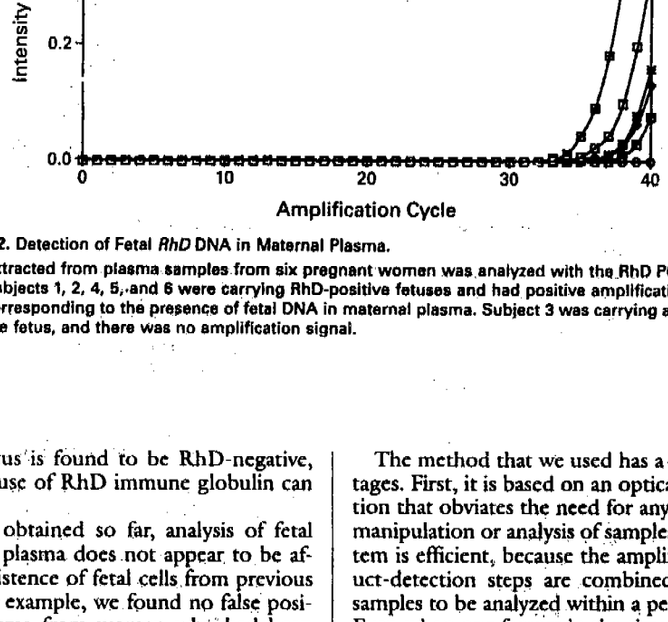

Figure 2. Detection of Fetal *RhD* DNA in Maternal Plasma.

DNA extracted from plasma samples from six pregnant women was analyzed with the RhD PCR system. Subjects 1, 2, 4, 5, and 6 were carrying RhD-positive fetuses and had positive amplification signals, corresponding to the presence of fetal DNA in maternal plasma. Subject 3 was carrying an RhD-negative fetus, and there was no amplification signal.

women. If the fetus is found to be RhD-negative, then unnecessary use of RhD immune globulin can be avoided.[22]

From the data obtained so far, analysis of fetal DNA in maternal plasma does not appear to be affected by the persistence of fetal cells from previous pregnancies.[23] For example, we found no false positive results in plasma from women who had been pregnant before and who were carrying RhD-negative fetuses in the current pregnancy. This finding is consistent with our previous data obtained using Y-chromosome–specific PCR testing: there were no false positive results in women who had previously been pregnant with a male fetus.[14]

Because of the high concentration of fetal DNA in maternal plasma,[14] the results of fetal genotyping of DNA extracted from maternal plasma are more reliable than those obtained by fetal genetic analysis of the cellular fraction of maternal blood. It also does not rely on the isolation of fetal cells, which requires the use of specialized, time-consuming, and technically demanding techniques such as cell sorting[24] and micromanipulation.[25] The high sensitivity of our PCR system is most likely the result of the use of an efficient protocol for the extraction of DNA and a fluorescence-based DNA system of amplification detection. Our current protocol for the extraction of DNA allows us to use eight times as much plasma DNA per amplification as was used in our previous study.[11]

The method that we used has a number of advantages. First, it is based on an optical system of detection that obviates the need for any postamplification manipulation or analysis of samples. Second, the system is efficient, because the amplification and product-detection steps are combined. This allows 96 samples to be analyzed within a period of two hours. Even when one factors in the time needed to extract DNA from plasma, this method of fetal genotyping can easily be performed in one day. The brevity of this method should facilitate efficient clinical decision making and decrease the time that sensitized RhD-negative women spend waiting to learn the RhD status of their fetuses.

The Rh family of polypeptides is encoded by two related genes: the *RhCE* gene and the *RhD* gene.[3,26] Because of the genetic complexity of the Rh system, several primer sets have been described for use in RhD genotyping.[5,6,27] The extent of agreement between the results of genotyping and serologic results is high, although the results can be discordant, possibly because of the existence of uncommon polymorphisms.[27]

Our findings indicate that the results of genotyping of fetal DNA extracted from maternal plasma are accurate and can potentially be used for the diagnosis of many disorders involving single genes. This approach may also be used to identify disorders such as cystic fibrosis and β-thalassemia in families in which the father and mother carry different mutations.[28]

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Drs. Lo and Wainscoat have applied for a patent for the RhD test procedure described in this paper.

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